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THE EFFECTS OF VANILLOID-LIKE AGENTS ON PLATELET AGGREGATION

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List of Abbreviations

AA	Arachidonic acid
APC	Activated protein C
ADP	Adenosine diphosphate
AEA	N-arachidonoyl-ethanol-amide
2-AG	2-Arachidonylglycerol
ATP	Adenosine triphosphate
AUC	Area under curve
CAP	Capsaicin
CGRP	Calcitonin gene-related peptide
CB	Cannabinoid receptors
COX	Cyclooxygenase
DHC	Dihydrocapsaicin
DRG	Dorsal root ganglia
ELISA	Enzyme-linked Immunosorbent Assay
FVII	Factor VII
FAAH	Fatty acid amide hydrolase
GPCRs	G protein-coupled receptors
GP	Glycoprotein
2-HPETE	12-Hydro-peroxyeicosatetraenoic
5-HT	5-Hydroxytryptamine
5-iodo-RTX	5-Iodo-resiniferatoxin
IP ₃	1,4,5- Inositol triphosphate
JAMs	Junctional adhesion molecules

LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
Max	Maximum aggregation
MAGL	Monoacylglycerol lipase
NADA	N-arachidonoyl-dopamine
OLDA	N-oleoyldopamine
OCS	Open canalicular system
PIP ₂	Phosphatidylinositol-4,5-bisphosphate
PLC	Phospholipase C
PLG	Plasminogen
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet derived growth factor
PECAM-1	Platelet endothelial cell adhesion molecule 1
PF4	Platelet factor 4
PRP	Platelet rich plasma
PPP	Platelet poor plasma
PAR	Protease-activated receptor
PKC	Protein kinase C
SD	Standard deviation
SEM	Standard error of the mean
β-TG	β-thromboglobulin
THC	Delta-9-tetrahydrocannabinol
TAFI	Thrombin-activatable fibrinogen inhibitor
TxA ₂	Thromboxane A ₂
TF	Tissue factor

TFPI	Tissue factor pathway inhibitor
tPA	Tissue plasminogen activator
TRPV1	Transient receptor potential vanilloids
TM	Transmembrane
uPA	Urokinase
vWF	Von Willebrand factor

Abstract

Capsaicin, the ‘hot’ principle found in chilli, and other vanilloids exert their effects on neuronal cells through activation of transient receptor potential vanilloid 1 (TRPV1). TRPV1 is widely distributed in neuronal and non-neuronal cells. It has been proposed that consumption of vanilloid-like agents, including capsaicinoids, inhibits platelet aggregation and may protect against the development of cardiovascular disease. The aim of this study was to investigate the effects of a range of vanilloid-like agents on *in vitro* platelet aggregation.

Venous blood was collected from healthy subjects who avoided antiplatelet medications and dietary chilli for at least 10 and 2 days, respectively. Collagen (4 and 8 $\mu\text{g/mL}$), ADP (10 and 5 μM) and arachidonic acid (AA) (300 and 400 mg/mL)-induced platelet aggregation was determined using platelet rich plasma (PRP; $250 \times 10^9/\text{L}$) in the absence and presence of the capsaicinoids [capsaicin and dihydrocapsaicin (DHC)] and the endocannabinoid/endovanilloid agents [N-oleoyldopamine (OLDA) and N-arachidonoyl-dopamine e (NADA)]. %Maximum aggregation (%Max), % area under curve (%AUC) and slope of platelet aggregation were determined. Platelet lactate dehydrogenase (LDH), which is released rapidly after cell membrane damage, was investigated to determine the direct toxic effects of these agents on platelets. Platelet factor 4 (PF4) and β -thromboglobulin (β -TG) release were examined to determine the effects of vanilloids on alpha granule release. Finally, the effects of TRPV1 antagonist (SB-452533) on capsaicin- and OLDA-mediated inhibition of ADP-induced platelet aggregation were investigated.

ADP-induced (5 μ M) platelet aggregation was inhibited in a concentration-dependent manner by capsaicin (%Max, mean \pm SEM; 0 vs 100 μ M, 83.8 \pm 0.9% vs 45.2 \pm 2.4%, n=6, p<0.001); OLDA (0 vs 100 μ M, 71.6 \pm 8.2% vs 9.4 \pm 1.4%, n=4, p<0.001); and NADA (0 vs 100 μ M, 71.5 \pm 5.9% vs 38.2 \pm 1.4%, n=4, p<0.008). Similar results were observed using 10 μ M ADP. OLDA and NADA, but not capsaicin and DHC, inhibited platelet aggregation induced by 4 μ g/mL collagen: OLDA (Max%, 0 vs 100 μ M, 89.3 \pm 1.4% vs 45.5 \pm 12.5%, p<0.001); and NADA (0 vs 100 μ M, 87.7 \pm 0.8% vs 28.5 \pm 8.2%, p<0.001). AA-induced (300 mg/mL) aggregation was inhibited in a concentration-dependent manner by capsaicin (Max%, 0 vs 100 μ M, 89.6 \pm 0.9% vs 11 \pm 0.8%, p<0.001); DHC (0 vs 100 μ M, 88.3 \pm 2.1% vs 18.7 \pm 6.9%, p<0.001); and NADA (0 vs 100 μ M, 84 \pm 1.8% vs 21.9 \pm 4.7%, p<0.001). Similar results were observed using 400mg/mL AA. The inhibition of platelet aggregation by all agents was not due to direct toxic effects as LDH release from platelets was unaffected by any of the vanilloids. SB-452533 did not inhibit the effects of OLDA (SB-45253; Max 0 vs 10 μ M, 55.9 \pm 2.1% vs 58.4 \pm 1.37%) and capsaicin (SB-45253; Max 0 vs 10 μ M, 65.15 \pm 0.44% vs 65.55 \pm 1%) on platelet aggregation, suggesting that inhibition of ADP-induced aggregation is not TRPV1 mediated. ADP-stimulated PF4 release from platelets was impaired by capsaicin, DHC and OLDA whereas NADA enhanced ADP-stimulated PF4 release. Furthermore, OLDA and capsaicin impaired the release of β -TG from ADP-stimulated platelets.

The present study using human platelet shows that capsaicin, DHC, OLDA and NADA inhibit *in vitro* aggregation. The inhibitory effects of vanilloids are not TRPV1 mediated and not due to a direct toxic effect on platelets. Vanilloids may

inhibit platelet aggregation by interfering with granule release, although further investigation of this possibility is warranted.

Chapter 1 LITERATURE REVIEW

1.1 Introduction

Platelets play an essential role in cardiovascular diseases both in pathogenesis of atherosclerosis and in the development of acute thrombotic events (Harker et al., 1976, Zucker, 1980). Their importance in coronary disease and in acute coronary syndromes is indirectly confirmed by the benefit of antiplatelet agents in treating these disorders. Several research groups have studied natural compounds as potential antiplatelet agents. One of the exciting discoveries from these studies was that the active ingredient of the hot chilli pepper, capsaicin, inhibits *in vitro* platelet aggregation (Adams et al., 2009, Hogaboam and Wallace, 1991, Raghavendra and Naidu, 2009).

The mechanism(s) by which capsaicin inhibits platelet aggregation is still poorly understood. To date only few studies have been conducted on human, rabbit and dog platelets to determine the effect of capsaicin and DHC on platelet aggregation and the mechanism(s) of its action(s) and those limited studies have produced conflicting data (Adams et al., 2009, Mittelstadt et al., 2012, Hogaboam and Wallace, 1991, Harper et al., 2009, Raghavendra and Naidu, 2009). The effects of both endogenous and plant-derived vanilloid-like agents on *in vitro* human platelet aggregation and the potential mechanism(s) of action were therefore systematically investigated in this study. The aims were to: (1) investigate the effects of exogenous and endogenous vanilloids on *in vitro* platelet aggregation, (2) determine whether vanilloid-like agents are toxic to platelets, (3) determine effect of vanilloid-like agents on platelet alpha and dense granule release, and (4) investigate the potential involvement of TRPV1 in mediating the anti-platelet effect of vanilloid-like agents.

1.2 Haemostasis

The haemostatic response to blood vessel damage includes a series of interactions between platelets, the subendothelial matrix and coagulation proteins. Under normal conditions, endothelial cells prevent tissue factor (TF) and the subendothelial matrix from being exposed directly to platelets and coagulation proteins. The contact between platelets and the subendothelial matrix induces platelet activation to facilitate plug formation. The haemostatic plug works as a catalytic surface for stimulation and recruitment of coagulation proteins, as well as localization and amplification of the coagulation system (Bombeli and Spahn, 2004, Hoffman, 2003, Hoffman and Monroe, 2001, Lawson and Murphy, 2004, Walsh, 2004). The haemostatic system is controlled by many anticoagulant proteins and inhibitors, as well as the fibrinolytic system. When working in balance, these systems ensure that the formed thrombus stops bleeding and that revascularization occurs afterward to maintain blood flow (Spahn and Rossaint, 2005).

1.2.1 Platelets

Platelets are small anuclear cytoplasmic fragments of megakaryocytes that are produced in the bone marrow (Avraham, 1993). Their major function is to maintain normal hemostasis and wound repair, but they also participate in many other pathophysiological processes such as atherosclerosis. Platelets possess many receptors that regulate platelet function (Ruggeri, 2002, De Botton et al., 2002).

1.2.1.1 Platelet Structure and Function

Platelets are small irregularly shaped cell fragments, with dimensions of approximately $3.0 \times 0.5 \mu\text{m}$, an average volume of 6-8 femtoliters (fL) and exhibit

heterogeneity in size and structure. The circulating number of platelets in the peripheral blood ranges from $150-400 \times 10^9/L$ (George, 2000). They play a major role in the physiological and pathological processes of hemostasis; wound healing, inflammation, host defense, and tumor metastasis (Jurk and Kehrel, 2005, Hoak, 1988). The main components of platelets are alpha (α) granules, dense granules, the dense tubular system, open canalicular system (OCS), cytoplasmic membrane, mitochondria, peroxisomes, lysosomes and cytoskeleton (Table 1-1) (Kamath et al., 2001, Fukami and Salganicoff, 1977).

Table 1-1 Contents of Platelet Granules (Kamath et al., 2001)

Electronic dense granules	Alpha granules	Lysosome	Peroxisomes
ADP (metabolically inactive storage form)	Fibrinogen	Hydrolytic enzymes	Catalase
Serotonin	Factor V		
ATP	Platelet derived growth factor (PDGF)		
Pyrophosphate	Platelet specific proteins		
Calcium	Platelet factor 4		
	β -thromboglobulin		
	Albumin		
	Fibronectin		
	Thrombospondin		

Platelets are full of secretory granules that are crucial for their normal function (Coppinger et al., 2004). Dense granules store small molecules including serotonin, calcium ions and nucleotides, particularly adenosine triphosphate (ATP) and adenosine diphosphate (ADP). These constituents induce localized vasoconstriction and cause activation of other platelets, and any defect in their release may affect normal haemostasis (Ren et al., 2008). Platelets also contain other structures such as peroxisomes, that store catalase, and lysosomes that contain hydrolytic enzymes such as hexosaminidase and cathepsins, that may have a role in further platelet activation and clot remodeling (Kamath et al., 2001, Ren et al., 2008, Anitua et al., 2004).

The most abundant platelet secretory granules are α -granules, accounting for ~10% of platelet volume, which is ten-fold more than dense granules. Furthermore, the total membrane surface area α -granules in a platelet is $14\mu\text{m}^2$, which is eight-fold more than dense granules and roughly equal to that of the open canalicular system. There are approximately 50-80 α -granules per platelet and their size ranges from 200-500nm (Frojmovic and Milton, 1982). Moreover, the role of α -granules in atherosclerosis, angiogenesis, host defense, inflammation, wound healing, malignancy, and antimicrobial have been well described (Table 1-2) (Blair and Flaumenhaft, 2009).

1.2.1.2 Platelet Activation

The process of platelet activation can be classified into three overlapping phases: initiation, extension and perpetuation (Hoffman et al., 2008). Although several agonists initiate platelet activation *in vitro*, *in vivo* activation is normally initiated by thrombin and collagen. Platelets can adhere to subendothelial matrix proteins such as collagen once the endothelium has been disrupted, with these proteins having an important role in platelet attachment and the amplification reaction (Table 1-3). The disrupted vessel wall captures and then activates platelets via exposed collagen attached to von Willbrand factor multimers, building a monolayer that aids thrombin generation and the subsequent aggregation of more platelets (Falati et al., 2003, Gross et al., 2005, Del Conde et al., 2005). Essential to these events are platelet surface receptors that assist the vWF-dependent binding of platelets, glycoprotein Ib/IX/V (GPIb-IX-V) and, to a lesser extent, integrin $\alpha_{\text{IIb}}\beta_3$ and subsequent intracellular signaling (through $\alpha_2\beta_1$ and GPVI), which leads to the attraction of

Table 1-2 Platelet Alpha Granule Contents and Functions

Functions	Factors	References
Chemokines	CXCL1 (GRO- α), CXCL4 (platelet factor 4), CXCL5 (ENA-78), CXCL7 (PBP, β -TG, CTAP-III, NAP-2), CXCL8 (IL-8), CXCL12 (SDF-1 α), CCL2 (MCP-1), CCL3 (MIP-1 α), CCL5 (RANTES)	(Brandt et al., 2000, Gleissner et al., 2008)
Growth factors	Vascular endothelium growth factor (VEGF), Platelet-derived growth factor (PDGF), Fibroblast growth factor (FGF), Epidermal growth factor (EGF), Hepatocyte growth factor (HGF), Insulin-like growth factor (IGF)	(Rendu and Brohard-Bohn, 2001, Nurden et al., 2008)
Angiogenesis inhibitors	TSP-1	(Jimenez et al., 2000)
Pro angiogenic mediators	Angiopoietin, CXCL12 (SDF-1 α), Matrix metalloproteinases (MMP-1, -2, and -9)	(Karshovska et al., 2007, Massberg et al., 2006)
Complement and complement binding proteins	Complement C3, Complement C4 precursor	(Maynard et al., 2007)
Adhesive receptors	GPIIb-IX-V (major receptor for fibrinogen), Integrin α IIb β 3, GPVI (collagen receptor)	(Berger et al., 1996, Suzuki et al., 2003)
Membrane bound proteins	Integrins (e.g., α IIb, α_6 , β_3), Immunoglobulin family receptors (e.g. GPVI, Fc receptors, PECAM), Leucine-rich repeat family receptors (e.g., GPIb-IX-V complex), Tetraspanins (e.g., CD9), Other receptors (CD36, Glut-3), P-selectin	(Suzuki et al., 2003, Nurden et al., 2004, Niiya et al., 1987, Maynard et al., 2007, Berger et al., 1993)
Coagulation factors and co-factors	Factors V, Factors XI, Factor XIII	(Rendu and Brohard-Bohn, 2001, Hayward et al., 1995, Jeimy et al., 2008, Kiesselbach and Wagner, 1972)
Inactive precursor	Thrombin, Prothrombin, High molecular weight kininogens, Plasminogen	(Maynard et al., 2007, Rendu and Brohard-Bohn, 2001)
Inhibitory proteases	Plasminogen activator inhibitor-1 (PAI-1), α_2 -antiplasmin	(Rendu and Brohard-Bohn, 2001)
Anticoagulants	Antithrombin, C1-inhibitor	(Blair and Flaumenhaft, 2009)
Adhesion molecules	Fibrinogen, Von Willebrand factor (vWF), Fibronectin, Vitronectin, Thrombospondin	(Cramer et al., 1988, Gralnick et al., 1985)

Table 1-3 Major Sub Endothelial Matrix Constituents that support Platelet Adhesion

(Hoffman et al., 2005b)

Matrix Constituent	Comment
Collagen	Large family of proteins with certain members supporting platelet adhesion, aggregation, and secretion
von Willbrand factor	Large multimeric protein critical for the hemostatic function of platelet
Fibronectin	Dimeric or multimeric protein that supports attachment and spreading of platelets
Thrombospondin-1	Trimeric protein exhibiting both adhesive an anti-adhesive properties
Laminins	Protein supporting platelet attachment
Microfibrils	A fibular bundle of protein constituents found in certain matrices

further platelets to extend on the endothelium and form a nidus for subsequent platelet-platelet interactions (Massberg et al., 2003).

At the site of vessel injury, collagen receptors help the capture of fast-moving platelets, resulting in activation of platelets and reorganization of the cytoskeleton. Platelets flatten out and attach more closely to the exposed vessel wall. vWF enhances this event by increasing the binding site affinity of collagen for platelets (Massberg et al., 2003, Kato et al., 2003, Poole et al., 1997, Nieswandt et al., 2001, Nieuwenhuis et al., 1985, Sixma et al., 1997). In inflammatory and thrombotic diseases, platelet activation is initiated by thrombin through G protein-coupled receptors (GPCRs) of the protease-activated receptor (PAR) family. The initiation step is sufficient to form a platelet plug but not to prevent bleeding (Hoffman et al., 2008).

The second stage of platelet aggregation occurs when more platelets are recruited, activated and aggregate on top of the collagen-bound monolayer (Hoffman et al., 2008). The secretion of agonists, such as thromboxane A₂ (TxA₂), thrombin and ADP, recruit further platelets to the site of injury and activate phospholipase C (PLC). The PLC isoform, PLC γ 2, hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP₂) to form 1,4,5- inositol triphosphate (IP₃) and diacylglycerol. IP₃ raises the cytosolic Ca²⁺ concentration by opening Ca²⁺ channels in the platelet-dense tubular system. This leads to Ca²⁺ influx through the plasma membrane of the platelet (Nesbitt et al., 2003, Kulkarni et al., 2004). The most important cohesive interaction that maintains the adhesion between the platelets is the binding of vWF or fibrinogen to $\alpha_{IIb}\beta_3$. Locally secreted or circulating catecholamines result in vasoconstriction and enhance platelet activation by increasing the effects of other platelet agonists. The majority of platelet agonists exert their action to extend the platelet plug through GPCRs. The characteristics of GPCRs make them especially well-suited for this function. Human platelets have almost 10 forms of G α , which fall into the G_{12 α} , G_{i α} , G_{s α} and G_{q α} groups (Offermanns, 2006).

The last stage of perpetuation occurs when the platelet plug is stabilized to prevent premature disaggregation (Hoffman et al., 2008). The interactions between platelets can be indirect, for example polyvalent adhesive proteins bind to activate $\alpha_{IIb}\beta_3$ on other platelets, or direct, where one cell adhesion molecule links to adjacent platelet in *trans*. Theoretically, both mechanisms support an additional adhesive force and another source of intracellular signaling (Brass et al., 2005). The binding between $\alpha_{IIb}\beta_3$ and fibrin, fibrinogen or vWF provides the main cohesive strength that stabilizes the aggregated platelet (Shattil and Newman, 2004). Other adhesive

molecules aside from integrins support the adhesion and intracellular signaling such as, platelet endothelial cell adhesion molecule 1 (PECAM-1; CD31) (Newman and Newman, 2003), junctional adhesion molecules (JAMs) (Muller, 2003, Bazzoni, 2003) and signaling lymphocytic activation molecule (SLAM; CD150) (Krause et al., 2000, Martin et al., 2001, Nanda et al., 2005). Platelet activation is thus a dynamic process where effector pathways and many receptors are controlling at each phase of platelet plugs (Hoffman et al., 2008).

1.2.1.3 Platelet Receptors

Several nomenclature systems have been used to classify platelet membrane proteins (Table 1-4) (Hoffman et al., 2008). The majority of platelet adhesive proteins belong to the integrin family, a broadly distributed group of heterodimeric cell surface molecules (having two subunits, α and β). Eight β -subunits are known that display high sequence homology, ranging from 35% to 45% at the first amino acid sequence level and a common structural organization. The α -subunits are also similar but show less extensive sequence identity (Hynes, 1992, Fitzgerald et al., 1987). Every β -subunit joins in a noncovalent complex with an α -subunit to make an efficient adhesive protein receptor. A solitary β -subunit can combine with many α -subunits. The β_1 and β_3 (β_2 is reported to be present at low levels) are the major β -subunits expressed on platelet, as well five α -subunits (Table 1-5) (Philippeaux et al., 1996). The $\alpha_{IIb}\beta_3$ integrin (GPIIb/IIIa) is restricted predominantly to platelets and megakaryocytes and plays a major role in platelet aggregation and several other platelet reactions (Grossi et al., 1988, Honn et al., 1992, Boukerche et al., 1989). GPIb-V-IX however, is not a member of the integrin family but is involved in platelet adhesion (Andrews et al., 2003). The major role of GPIb-V-IX in

Table 1-4 Platelet Receptors for Adhesive Protein (Hoffman et al., 2005b)

Ligand	Receptor (s)	Other Common Designation(s)
Collagen	GPIa/IIa, $\alpha 2\beta 1$ GPIIb/IIIa, $\alpha \text{IIb}\beta 3$ GPIV GPVI	VLA-2 GPIIb, CD36
Fibrinogen	GPIIb/IIIa, $\alpha \text{IIb}\beta 3$	
Fibronectin	Vitronectin receptor, $\alpha \nu \beta 3$ GPIc/IIa, $\alpha 5\beta 1$ GPIIb/IIIa, $\alpha \text{IIb}\beta 3$	VLA-5
Thrombospondin	Vitronectin receptor, $\alpha \nu \beta 3$ GPIV Integrin-associated protein	GPIIb, CD36 IAP
Vitronectin	Vitronectin receptor, $\alpha \nu \beta 3$ GPIIb/IIIa, $\alpha \text{IIb}\beta 3$	
von Willebrand factor	GPIb/IX GPIIb/IIIa, $\alpha \text{IIb}\beta 3$	
Laminin	GPIc/IIa region, $\alpha 6\beta 1$	VLA-6

Table 1-5 Platelet Integrins (Hoffman et al., 2005b)

Integrin	Major Ligand (s)
$\alpha 2\beta 1$ (GPIa/IIa, VLA-2)	Collagen
$\alpha 5\beta 1$ (GPIc/IIa, VLA-5)	Fibronectin
$\alpha \text{IIb}\beta 3$ (GPIIb/IIIa)	Fibrinogen, fibronectin, vitronectin, von Willebrand factor, CD40L
$\alpha \nu \beta 3$ (vitronectin receptor)	Fibrinogen, fibronectin, vitronectin, von Willebrand factor, thrombospondin, osteopontin
$\alpha 6\beta 1$ (GPIc/IIa region)	Laminin

haemostasis can be traced directly to its function as the vWF receptor as well as a binding site for thrombin (Moroi et al., 1982, Okumura and Jamieson, 1976). Therefore, by adhesion with matrix proteins, vWF mediates directly reversible and rapid platelet adhesion that supports the rolling of platelets along the surface of the disrupted endothelium (Sadler, 2002). The most likely receptors responsible for the firm platelet-adhesive bond that enduringly stops the rolling of the platelet are integrins. A significant consequence of occupancy of GPIb-V-IX by vWF is the stimulation of intracellular signaling events that lead to the activation of $\alpha_{IIb}\beta_3$ and platelet aggregation (Berndt et al., 2001, Savage et al., 1992).

Collagen in the subendothelial matrix is an important initiator of platelet responses. It is both a substrate for platelet adhesion and a potent platelet agonist. Platelets have four types of collagen receptors. Two bind directly to collagen ($\alpha_2\beta_1$ and GPVI), and two bind to collagen through vWF ($\alpha_{IIb}\beta_3$ and GPIb-IX-V). GPVI acts as the primary collagen receptor and is responsible for platelet secretion and aggregation induced by collagen. In addition, $\alpha_2\beta_1$ works as an anchor for platelets to adhere to exposed collagen after endothelial injury (Hoffman et al., 2005a, Hoffman et al., 2008, Clemetson et al., 1999).

ADP is another platelet agonist that causes shape change of platelets from smooth discoid shape to speckled spheres, liberation of granule contents and release of thromboxane A₂, ultimately causing platelet aggregation (Jin et al., 1998). ADP is released actively from the platelet dense granules in response to the physiological platelet agonists, which are thrombin, TXA₂ and collagen, and amplifies its own effects as well as those of other activators, by a positive feedback mechanism

(Shankar et al., 2006, Maffrand et al., 1988, Born, 1985). Damaged vessel walls (endothelial cells) and erythrocytes also release ADP passively, which induces aggregation of platelets through integrin $\alpha_{IIb}\beta_3$ activation and subsequent fibrinogen binding (Mills, 1996). ADP mediates platelet aggregation via binding to two G-protein-coupled receptors subtype, P2Y1 and P2Y12 (Figure 1-1) (Jantzen et al., 1999, Daniel et al., 1998). Activation of P2Y1 is sufficient to cause platelet shape change, while co activation of both P2Y1 and P2Y12 is required to induce platelet aggregation (Paul et al., 1999, Jin et al., 1998, Jin and Kunapuli, 1998, Savi et al., 1998).

The P2Y1 receptor is coupled to the heterotrimeric protein Gq. P2Y1 activation leads to activation of phospholipase C, production of diacylglycerol and inositol phosphate (IP). Moreover, calcium mobilization from cytosolic stores in response to IP formation results in activation of protein kinase C (PKC) and phosphorylation of myosin light chain, signaling events that play a major role in agonist-induced platelet shape change (Daniel et al., 1998, Daniel and Adelstein, 1976, MacKenzie et al., 1996). ATP weakly antagonizes these effects of ADP at the P2Y1 receptor, in comparison to strong P2Y1 receptor-specific adenine nucleotide analog antagonists, A3P5PS, A3P5P, and A2P5P. These three antagonists are required in high concentrations (20-fold molar excess) to block functional responses to 10mM ADP, while a 200-fold molar excess of ATP is required to completely inhibit the P2Y1 receptor (Boyer et al., 1996, Eckly et al., 2001). The absence of P2Y1 in mouse models of thrombosis has been shown to increase survival after the administration of platelet agonists and TF (Woulfe et al., 2001a, Leon et al., 1999, Leon et al., 2001).

The second ADP receptor, P2Y₁₂, is coupled to G_{i2} and its activation results in inhibition of cAMP formation by adenylyl cyclase (Hollopeter et al., 2001, Zhang et al., 2001). Lack of P2Y₁₂ in humans produces a relatively mild hemorrhagic phenotype (Hollopeter et al., 2001, Cattaneo and Gachet, 1999, Nurden et al., 1995). Moreover, deletion of either P2Y₁ or P2Y₁₂ in mice prolongs bleeding time and impairs the response of platelets to TxA₂, ADP and thrombin, especially at low concentrations (Foster et al., 2001, Fabre et al., 1999, Leon et al., 1999).

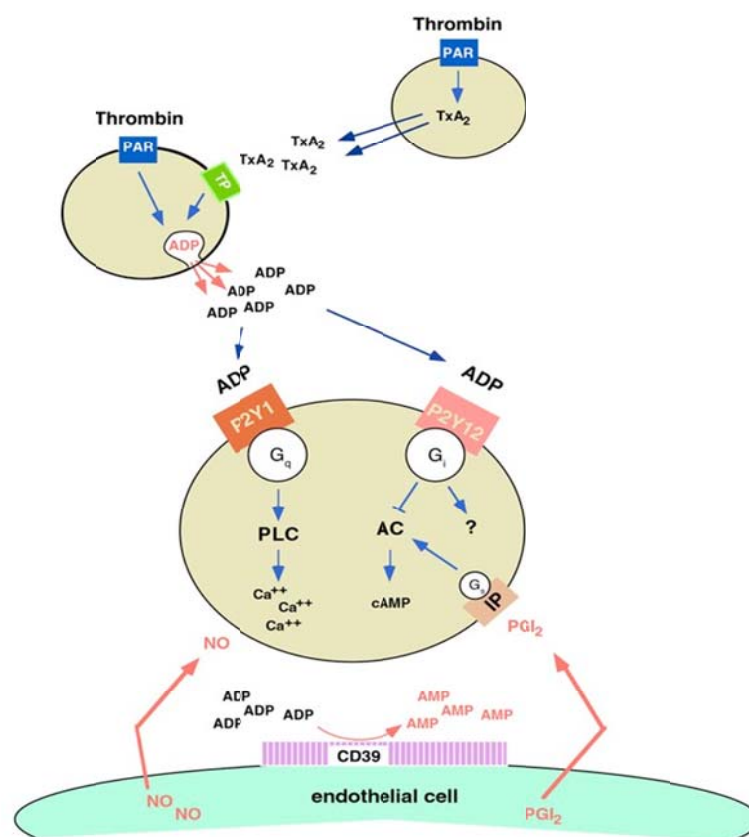


Figure 1-1 ADP and Platelet Activation

Platelet activation by potent agonists such as thrombin or collagen causes the release of secondary agonists such as thromboxane A₂ (TxA₂) and the secretion of ADP from platelet dense granules. Platelet responses to ADP require the coordinate activation of two G protein-coupled receptors, P2Y₁ and P2Y₁₂, whose actions are described in the text. Drugs such as, Ticlopidine and Clopidogrel block activation of P2Y₁₂. Mechanisms that place a limit on unwarranted platelet activation include CD39 on the surface of endothelial cells, which hydrolyzes ADP to AMP, and PGI₂ and NO, which increase the concentration of cAMP and cGMP within platelets (Woulfe et al., 2001b)

1.2.2 Blood Coagulation

The blood coagulation system contributes to the stabilization of the primary platelet plug. Natural endogenous anticoagulants regulate blood coagulation to ensure that, under normal conditions, haemostasis remains balanced. Any disturbance of this balance between coagulation and anticoagulation due to acquired or genetic factors may cause bleeding or thrombosis (Dahlback, 2000).

1.2.2.1 Tissue Factor Pathway

Blood coagulation is initiated by the exposure of TF, which is present in the cells that encircle the vascular bed as well as expressed by cells circulating in the blood such as leukocytes. TF binds to factor VII (FVII) and the activated form of FVII (FVIIa). The TF-FVIIa complex triggers the conversion of FIX and FX to FIXa and FXa respectively (Kirchhofer and Nemerson, 1996, Mann et al., 1998). FIXa and FXa remain attached to the TF-bearing cell or diffuses into the blood to adhere to activated platelets (Hoffman et al., 1996). Platelet activation is combined with the disclosure of negatively charged phospholipids which have high affinity to bind and assemble enzyme-cofactor complex and coagulation factors that are essential for effective propagation of the system (Zwaal et al., 1998).

The prothrombinase complex, which is a phospholipid-bound complex composed of FV and FXa, activates prothrombin to thrombin. Thrombin is a key enzyme in the coagulation system due to its multiple roles including platelet activation, fibrinogen to fibrin conversion and feedback amplification of coagulation (Figure 1-2). Thrombin activates FV, FVIII and FXI as feedback amplification. Another amplification loop is activation of FX by thrombin resulting in additional generation

of factor IXa that can lead to activation of factor X. vWF is an important adhesive protein for the creation of the primary platelet plug and FVIII circulates bounding to this factor. FVIII become activated and dissociated from vWF and make a complex with FIXa on the platelet surface, which eventually leads to FX activation (Gailani and Broze, 1991). Following fibrin clot formation, thrombin generation reaches its peak (Mann et al., 1998), which is important for the activation of FXIII and thrombin-activatable fibrinolysis inhibitor (TAFI) as well as for further fibrin generation (Davie, 1995).

Thrombin generation is considered to be more important than fibrin deposition due to the fact that thrombin plays an important role in the activation of platelets, which is a crucial reaction for haemostasis response to vascular injury (Dahlback, 2000).

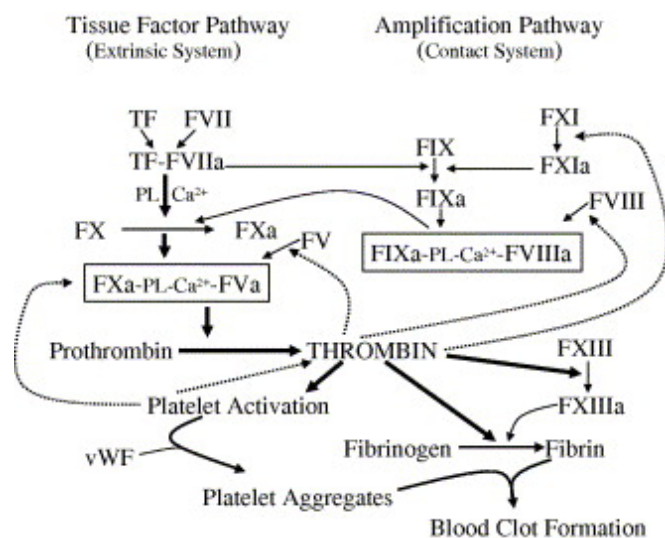


Figure 1-2 Schematic Diagram of the Coagulation Cascade

The initiation of blood clot formation occurs following vascular injury and the exposure of tissue factor (TF) to circulating blood. Thrombin exerts positive feedback regulation (dotted lines) by activating platelets and other procoagulant proteins. Activated platelets provide a phospholipid (PL) surface that serves to enhance enzyme complex formation. Platelet aggregates are anchored to damaged endothelium by von Willebrand Factor (vWF) (Gentry, 2004)

1.2.2.2 Natural Inhibitors of Blood Coagulation

The coagulation system is controlled by three natural anticoagulants, the protein C anticoagulant system, tissue factor pathway inhibitor (TFPI) and antithrombin (Hoffman et al., 2008). Protein C is activated by thrombin and its cofactor thrombomodulin, to form activated protein C (APC). Thrombin alone is inefficient in activating protein C without its cofactor thrombomodulin; this complex increases the rate of protein C activation by 1000-fold. The protein C system inhibits the coagulation by controlling the activities of FVa and VIIIa, cofactors of the prothrombinase and tenase complexes, respectively (Dahlback and Villoutreix, 2005, Esmon, 2003, Nicolaes and Dahlback, 2002). Protein S and FV stimulate the activity of APC. Protein S is enough to inhibit activated factor V, while for regulation of activated factor VIII needs a synergistic APC cofactor function of both factor V and protein S (Nicolaes and Dahlback, 2002).

TFPI is the major inhibitor of TF-VIIa complex and plays an important role in regulating thrombin generation (Broze, 1995). TFPI inhibits activated factor VII by two mechanisms. Firstly, TFPI binds to and inhibits activated factor X that has been activated via TF-activated factor VII. Secondly, the complex of TFPI-activated factor X binds to TF-activated factor VII building an inactive quaternary complex (Hoffman et al., 2008). Heparin binds to antithrombin (AT) causing conformational change, which makes AT more reactive to thrombin, activated factor X and activated factor IX (Silverman et al., 2001, Lane et al., 2005, Carrell et al., 1991, Schulze et al., 1994).

1.2.3 Fibrinolysis

Fibrinolysis helps to balance coagulation to ensure blood flow and prevent bleeding (Degen, 2001, Esmon et al., 1999, Hajjar, 2003, Kolev and Machovich, 2003, Cesarman-Maus and Hajjar, 2005). Tissue plasminogen activator (tPA) and urokinase (uPA) are the major endogenous activators of fibrinolysis (Kasai et al., 1985, Pennica et al., 1983). tPA and uPA convert the circulating plasma zymogen [plasminogen, (PLG)] to plasmin, which is the main fibrinolytic protease (Holvoet et al., 1985). Fibrin enhances plasmin generation by binding PLG and tPA on its surface to protect it against the inhibitor, α_2 -antiplasmin. The affinity between tPA and PLG is reduced in the absence of fibrin but increased in its presence. tPA has much higher affinity for fibrin than uPA, but uPA is an effective PLG activator in the presence and the absence of fibrin (Gurewich et al., 1984, Lijnen et al., 1986).

α_2 -Antiplasmin is the major plasmin inhibitor, which immediately neutralizes plasmin in the flowing blood or in the vicinity of a platelet-rich thrombus (Holmes et al., 1987). In addition, plasmin activity is inhibited by α_2 -macroglobulin, but to a lesser extent compared to α_2 -antiplasmin (Aoki et al., 1978). Plasminogen activator inhibitor-1 (PAI-1) is the most important and rapidly acting inhibitor of tPA and uPA (Ny et al., 1986, Samad et al., 1996). Activated thrombin-activatable fibrinolysis inhibitor (TAFI) is a potent attenuator of fibrinolysis (Eaton et al., 1991, Nesheim, 2003). There are two kinds of fibrinolytic receptors, activation and clearance receptors. These receptors include $\alpha_{IIb}\beta_3$, integrin $\alpha_M\beta_2$, α -enolase, annexin 2, Heymann nephritis antigen and amphoterin which are expressed on platelets, leukocytes, monocytoïd cells, endothelial cells, renal epithelial cells and neuroblastoma cells respectively (Hajjar, 1995).

1.3 Cannabinoid Receptors

The endocannabinoid system is composed of the cannabinoid receptors (CB), endogenous cannabinoids (endocannabinoids) and enzymes that degrade and synthesis endocannabinoids (Howlett et al., 2002, Freund et al., 2003, Mackie, 2006). Endocannabinoid effects are mostly mediated by two G protein-coupled receptors (GPCRS), CB1 and CB2, even though other receptors might be involved such as TRPV1 (Howlett et al., 2002). CB1 receptors are mainly present in several brain regions and peripheral nerves and mediate retrograde synaptic inhibition of endocannabinoids (Herkenham et al., 1991, Matsuda et al., 1993, Marsicano and Lutz, 1999). CB2 receptors have a limited distribution, being present in some immune cells, some neurons and inflammatory cells (Galiegue et al., 1995, Van Sickle et al., 2005, Ofek et al., 2006).

In addition, CB1 and CB2 receptors are present in platelets but their role in platelet activation is unclear. Delta-9-tetrahydrocannabinol (THC) is a main compound of marijuana (natural cannabis) and it activates platelets by increasing P selectine and glycoprotein IIb-IIIa expression and not through activation of CB1 or CB2 (Deusch et al., 2004). Furthermore, it increases 2-arachidonylglycerol (2-AG) levels, resulting in activation of platelets through the AA pathway (Nakahata, 2008). A further study has shown that anandamide activates rabbit platelets through the same pathway (Braud et al., 2000). Both studies showed that it is not CB1 or CB2 mediated. On the other hand, it has been reported that anandamide (arachidonoyl ethanolamide, AnNH) activates human platelets through a mechanism independent of the AA pathway (Maccarrone et al., 1999). Later, the same group showed that 2-AG mediated aggregation via an uncharacterized CB receptor (MacCarrone et al., 2001).

CB1 and CB2 are similar to other GPCRs, depending on pharmacological influences, such as functional selectivity (Breivogel and Childers, 2000, Prather et al., 2000, Mukhopadhyay and Howlett, 2005, Urban et al., 2007), partial agonism (Straiker and Mackie, 2006) and inverse agonism (Vasquez and Lewis, 1999, Kenakin, 2001, Kenakin, 2007), which play a significant role in regulating the cellular response to specific cannabinoid receptor ligands (Mackie, 2008).

1.3.1 Endogenous Cannabinoids (Endocannabinoids)

Endocannabinoids include ‘endovanilloids’ mentioned later, such as the AA derivatives and eicosanoid family members, 2-arachidonylglycerol (2-AG), N-arachidonoyl-dopamine (NADA), anandamide (N-arachidonoyl-ethanol-amide, AEA), O-arachidonoyl-ethanolamine (virodhamine) and 2-arachidonoylglyceryl-ether (noladin ether) (Figure 1-3) (Blankman et al., 2007). In comparison to other chemical signals in the brain, endocannabinoids are not produced and stored in the neural cells but synthesized on demand from their precursors and then released (Simon and Cravatt, 2006, Alexander and Kendall, 2007, Basavarajappa, 2007, Okamoto et al., 2007).

Anandamide shares with capsaicin the ability to stimulate TRPV1. The main endocannabinoids, anandamide and 2-AG, are produced in response to an increasing concentration of intracellular calcium by diacylglycerol lipase, and are degraded and hydrolyzed by two enzymes, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Blankman et al., 2007, Bisogno, 2008). 2-AG hydrolysis produces AA that is the main substrate for cyclooxygenase (COX)

enzymes, which in turn convert AA into prostaglandins and thromboxanes (Nakahata, 2008). Moreover, 2-AG induces platelet aggregation not via activation of CB1 receptors but through activation of the COX pathway. CB1 and CB2 antagonism have no effect on 2-AG- induced platelet aggregation (Keown et al., 2010).

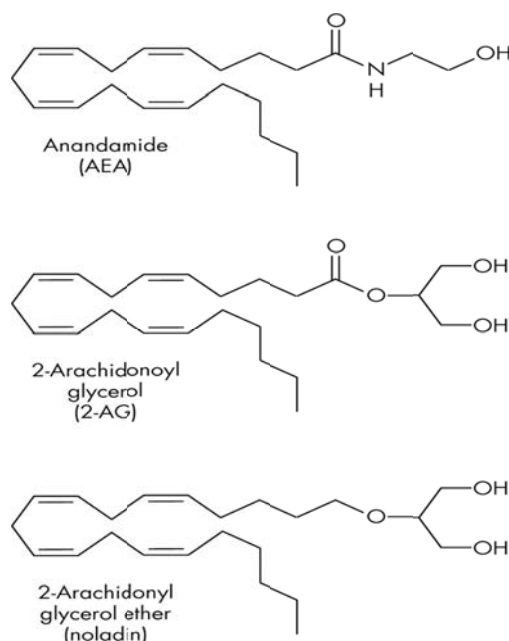


Figure 1-3 Chemical Structures of Endogenous Cannabinoids
Anandamide (AEA), 2-arachidonoyl glycerol (2-AG), and 2-arachidonoyl-glycerol ether (noladin) (Tomida et al., 2004)

1.4 Transient Receptor Potential Vanilloid Channels

The transient receptor potential vanilloid (TRPV) channel family is composed of six subtypes, TRPV1-6. The proteins with most similar homology at the amino acid level to TRPV1 are TRPV3 (43%), TRPV2 (46%) and TRPV4 (43%). However, TRPV1 is the only receptor that truly deserves the vanilloid receptor name, because it is the only member activated by capsaicin (the active constituent of ‘hot’ chili peppers) and its potent relative, resiniferatoxin (RTX) (Roberts et al., 2004).

1.4.1 TRPV1 Structure

TRPV1, or the capsaicin receptor, is a non-selective cation channel containing six transmembrane (TM) domains and a short, pore-forming hydrophobic stretch between the fifth and sixth TM domains (Figure 1-4) (Caterina et al., 1997). It is stimulated by a wide variety of agents, including plant-derived vanilloids (e.g. capsaicin and RTX), endogenous vanilloid-like lipids (e.g. [N-oleoyldopamine (OLDA) and NADA], in addition to heat ($> 43^{\circ}\text{C}$) and acid ($\text{pH} < 7$) (Caterina and Julius, 2001, Zhong and Wang, 2008). Activation of TRPV1 on sensory neurons leads to burning and painful sensations (Szallasi and Blumberg, 1999). Binding sites for capsaicin, which is lipophilic in nature, appear to be present on both sides of plasma membrane, as patch clamp studies have shown that capsaicin produces identical responses when added to either side of a patch, consistent with the notion that capsaicin can cross the lipid bilayer to mediate its effects (Caterina et al., 1997).

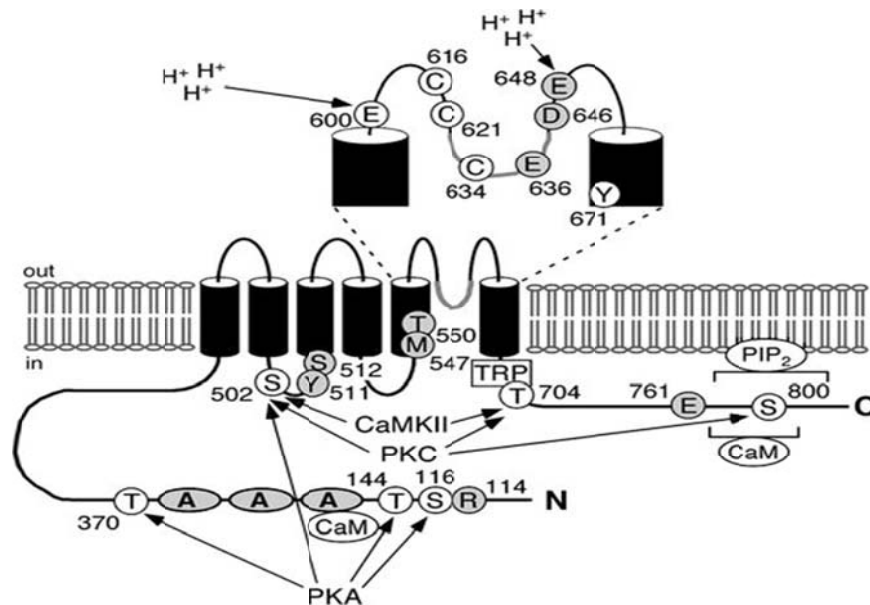


Figure 1-4 Regions and Amino Acids Involved in TRPV1 Function

Residues reported to be involved in vanilloid binding are shown in grey. TRP in a box indicates a TRP domain. Phosphatidylinositol (4,5)-bisphosphate (PIP_2) binds to the indicated region in the carboxyl (C) terminus. Calmodulin (CaM) binds to both C- and amino (N)-termini. A indicates the first ankyrin repeat. Protein kinases A (PKA) or C (PKC) or CaM kinase II (CaMKII) phosphorylate overlapping Ser (S) or Thr (T) residues indicated by arrows. Protons act on the two Glu (E) in the extracellular loop indicated by arrows (Tominaga and Tominaga, 2005)

1.4.2 TRPV1 Function

There are several amino acids and amino acid sequences of the TRPV1 protein that have defined functions, such as mediating capsaicin action, heat activation, phosphorylation and modulation by lipids, multimerization, proton action, permeability and desensitization. TRPV1 is therefore fundamental in peripheral nociception. Understanding the sequences and amino acids of TRPV1 could lead to the development of anti-nociceptive or anti-inflammatory agents (Tominaga and Tominaga, 2005).

1.4.2.1 Capsaicin Action

Capsaicin is structurally related to endogenous TRPV1 agonists, such as N-arachidonyl dopamine (NADA), 12-hydro-peroxyeicosatetraenoic (12-HPETE) and anandamide (Huang et al., 2002, Hwang et al., 2000, Zygmunt et al., 1999). Capsaicin and its congeners, for example RTX, are lipophilic, which allows them to pass through the cell membrane and act on binding sites on the intracellular domain of TRPV1, providing a possible explanation for the lag time between capsaicin intake and pungent sensation (Jung et al., 1999). TRPV1 has a similar structure to voltage-gated K^+ channels, involving the six-TM topology. As specified by the contemporary helix-packing models of the voltage-gated K^+ channels, the first, second and third TM domains are placed on the lipid-facing side of the tetrameric channel complex. In contrast, the fifth and sixth TM domains are placed nearer to the pore-forming channel core. Presuming TRPV1 is similar to helix packing, the capsaicin lipophilic moiety might bind to the second and third TM domains on the channel-lipid interface. The vanilloid moiety might interact with residues around Tyr 511 in the cytosolic region, therefore binding the cytosolic tail with two TM

domains (Kuzhikandathil et al., 2001, Tominaga and Tominaga, 2005, Welch et al., 2000).

1.4.2.2 Heat Activation

TRPV1 heat-evoked currents exhibit similar characteristics to those of capsaicin-evoked currents. However, the TRPV1 responses to heat and capsaicin are different, although the mechanisms overlap. Several TRP family ion channels (TRP (melastatin) 8 (TRPM8), TRP subfamily A member 1 (TRPA1), TRPV1, TRPV2, TRPV3, TRPV4) are thermosensitive, suggesting that the domains of temperature sensor are present in these protein channels (Jordt et al., 2003, Patapoutian et al., 2003, Tominaga and Caterina, 2004). TRPV1 displays the property of voltage-dependent gating, when it is activated by changes in temperature and depolarization, and results in graded shifts in its voltage-dependent activation curve (Gunthorpe et al., 2000).

1.4.2.3 Phosphorylation

TRPV1 is phosphorylated by kinases including protein kinase C (PKC), protein kinase A (PKA) and Ca^{2+} / Ca^{2+} M-dependent kinase II (CaMKII). Inflammatory mediators such as prostaglandins activate PKA-dependent pathway, which affects heat- or capsaicin- mediated effects on sensory nerve by acting on TRPV1 (Bhave et al., 2002, Mohapatra and Nau, 2003). In addition, it has been reported that PKA phosphorylates the Ser 116 and Thr 370 in the amino terminus, which leads to TRPV1 desensitization. Ser 116 phosphorylation by PKA suppresses TRPV1 dephosphorylation induced by capsaicin exposure (Rathee et al., 2002).

TRPV1 receptor PKC-dependent phosphorylation results from activation of Gq-coupled receptors via many inflammatory mediators including prostaglandins, ATP, bradykinin and tryptase or trypsin (Dai et al., 2004, Moriyama et al., 2003, Moriyama et al., 2005, Sugiura et al., 2002, Tominaga et al., 2001). PKC-dependent phosphorylation of TRPV1 decreases the temperature threshold for TRPV1 activation plus potentiates proton- or capsaicin-evoked responses, such that normally non-painful temperatures (normal range) are able to stimulate TRPV1 and lead to painful sensation (Numazaki et al., 2002). CaMKII regulates the activity of TRPV1 upon TRPV1 phosphorylation at Thr704 and Ser502 by controlling capsaicin binding. Therefore, TRPV1 phosphorylation via three different kinases appears to regulate the activity of the channel via the dynamic stability between dephosphorylation and phosphorylation processes (Jung et al., 2004).

1.4.2.4 Modulation by Lipids

Lipids derived from cell membranes are also known to regulate some ion channels, including TRPV1 (Ahern, 2003, Hwang et al., 2000, Zygmunt et al., 1999). Phosphatidylinositol-4,5-bisphosphate (PIP₂) seems to be associated with TRPV1, leading to ionic channel inhibition, while PIP₂-mediated inhibition appears to be discharged upon stimulation of PLC via metabotropic receptors, resulting in hydrolysis of PIP₂ to inositol (1,4,5) trisphosphate and diacylglycerol. PIP₂ deletion from TRPV1 upon hydrolysis by PLC or experimental sequestration results in channel activation (Chuang et al., 2001).

1.4.2.5 Multimerization

TRPV1, like many other TRP channels, has a carboxyl terminus consisting of a TRP domain near to the sixth TM and a long amino terminus consisting of three ankyrin-repeat domains (Sedgwick and Smerdon, 1999). The ankyrin repeat is composed of a ~33-residue motif labeled after ankyrin cytoskeletal protein, calmodulin (CaM) is a one protein that bind to the first ankyrin repeat domain of TRPV1 (Rosenbaum et al., 2004, Sedgwick and Smerdon, 1999). TRPV1 forms multimers with a homotetramer as the predominant form and heterooligomerises with TRPV3, which is another heat-sensitive TRP channel (Smith et al., 2002).

1.4.2.6 Proton Action

TRPV1 function is affected by acidification of the extracellular media in two ways. First, extracellular protons lower the threshold for TRPV1 channel activation, which increases the strength of capsaicin or heat as a TRPV1 agonist. Second, extracellular protons can act as an agonist by increasing the probability of opening the TRPV1 channel at room temperature with further acidification (to pH <6.0) (Tominaga et al., 1998). Mutational analysis has demonstrated that TRPV1 Glu 600, situated in extracellular putative domain, works as the main regulatory site for proton potentiation of TRPV1 activity, while Glu 648 is associated with direct proton-evoked TRPV1 activation (Jordt et al., 2000).

1.4.2.7 Permeability

The TRPV1 shows remarkable preference for bivalent cations, however the cation permeability region in TRPV1 is not well defined (Welch et al., 2000, Caterina et al., 1997). It exhibits high preference for Ca^{2+} with high relative permeability for Ca^{2+}

(Bevan and Szolcsanyi, 1990). Extracellular Ca^{2+} is an important factor in desensitization caused in presence of capsaicin (Holzer, 1991), therefore, it was found that elimination of extracellular Ca^{2+} in that it has decreased desensitization to capsaicin (Liu and Simon, 1996, Garcia-Hirschfeld et al., 1995). Moreover, any change in TRPV1 permeability to Ca^{2+} leads to loss of Ca^{2+} -dependent desensitization in the presence of extracellular Ca^{2+} (Mohapatra et al., 2003).

1.4.2.8 Desensitization

Capsaicin has paradoxical effects, in that it has algescic and analgesic action. These may relate to the ability of capsaicin to desensitize nociceptive terminals after prolonged exposure (Szallasi and Blumberg, 1999, Caterina et al., 1997). Desensitization to capsaicin is a complicated process with different kinetic components. These are a “rapid” component that depends on Ca^{2+} influx through TRPV1 and a “slow” component, which does not (Docherty et al., 1996, Koplas et al., 1997, Liu and Simon, 1996, Piper et al., 1999).

1.4.3 TRPV1 Distribution

TRPV1 is widely distributed throughout the peripheral nervous system, central nervous system and non-neuronal tissue. TRPV1 is highly expressed in more than 50% of human dorsal root ganglia (DRG) (Sanchez et al., 2001). It is more prevalent in small to medium sized neurons and TRPV1-like immunoreactivity has been demonstrated in thinly myelinated and unmyelinated fibers, conforming to the original hypothesis that TRPV1 was expressed mainly in nociceptors (Caterina and Julius, 2001). TRPV1 is also present in the spinal cord (mainly in sensory efferent fibers), several regions of the brain, such as substantia nigra, hippocampus and

hypothalamus (Sanchez et al., 2001), and non-neuronal tissues and cells such as bladder urothelium and smooth muscle (Birder et al., 2001), macrophages (Chen et al., 2003), liver hepatocytes (Reilly et al., 2003), pancreatic β -cells, endothelial cells, lymphocytes (Lai et al., 1998), epithelial cells lining human airways, keratinocytes (Southall et al., 2003) and polymorphonuclear granulocytes (Heiner et al., 2003). Furthermore, lung (Kollarik and Undem, 2004), dental pulp (Renton et al., 2003), urinary bladder (Yiangou et al., 2001b), gastrointestinal tract (GIT) (Ward et al., 2003) and prostate (Van der Aa et al., 2003), have fibres with TRPV1-like immunoreactivity, consistent with capsaicin-sensitive pathway distribution (Szallasi and Blumberg, 1999). Fluorescent labels have identified TRPV1 in more precise subcellular areas on the Golgi complex, cell membrane and smooth endoplasmic reticulum (Veronesi and Oortgiesen, 2006).

1.4.4 Biochemical Pharmacology of TRPV1

Expression of TRPV1 in DRG and its ability to mediate pain responses to vanilloids suggest that the expression of TRPV1 might be different in acute and chronic pain models in rat (Ji et al., 2002, Sanchez et al., 2001). It has been observed that the number of TRPV1-like immunoreactive fibres increases in the colon of patients with irritable bowel syndrome and active inflammatory bowel disease, and in rectal biopsy from patients with fecal urgency and rectal hypersensitivity (Chan et al., 2003, Yiangou et al., 2001a). Moreover, the number of TRPV1 receptors in DRG increased after DRG avulsion injury (e.g. central axotomy) (Smith et al., 2002). This information suggest that upregulated TRPV1 expression might be related to certain types of pathophysiologies that lead to pain, providing support for the therapeutic potential of TRPV1 antagonists (Cortright and Szallasi, 2004). Abnormal expression

of TRPV1 by neurons that do not express TRPV1 normally has been associated to the occurrence of inflammatory hyperalgesia and neuropathic pain (Hudson et al., 2001, Rashid et al., 2003).

Activation of TRPV1 in neurons and non-neuronal tissues results in rapid increases in intracellular Ca^{+2} levels. Cloned TRPV1 does not discriminate between monovalent cations, but exhibits significant preference for those that are divalent (sequence of permeability: $\text{Ca}^{+2} > \text{Mg}^{+2} > \text{Na}^{+} \approx \text{K}^{+} \approx \text{Cs}^{+}$). The relative permeability of TRPV1 is very high to Ca^{+2} ($P_{\text{Ca}}/P_{\text{Na}} = 9.60$; $P_{\text{Mg}}/P_{\text{Na}} = 4.99$), which exceeds what has been observed for most non-selective cation channels (Mayer and Westbrook, 1987, Seguela et al., 1993). In cultured sensory neurons, the continuous exposure to vanilloid leads to desensitization according to electrophysiological analyses of vanilloid-evoked response. However, in the absence of extracellular calcium, vanilloid-evoked responses showed little or no desensitization during continuous capsaicin application. Indeed, the response to capsaicin is depended on ambient calcium levels (Holzer, 1991, Liu and Simon, 1994).

1.5 Vanilloids

Capsaicinoids are the hot compounds in placental tissues of Capsicum fruits. They are responsible for capsicum's pungency by eliciting a sensation of burning pain by selectively activating sensory neurons that convey the noxious stimuli to the central nervous system (Caterina, Schumacher et al. 1997; Aza-Gonzalez, Nunez-Palenius et al. 2010).

1.5.1 Endogenous Vanilloids

TRPV1 is activated by endogenous AA derivatives that include the endocannabinoids, OLDA, NADA, N-acylethanolamines [N-oleoylethanolamine, anandamide, N-linoleoylethanolamine]; plus lipoxygenase products [e.g., leukotriene B₄, 12-(S)- and 15-(S)-hydroperoxyeicosatetraenoic]. These substances have different affinities to TRPV1 and are increasingly recognized as an important group of signaling molecules affecting tissue injury, pain and inflammation (Zhong and Wang 2008). The most potent and selective endogenous TRPV1 agonist is OLDA, which is 30 times more potent than capsaicin, and 50 times more potent at TRPV1 than at CB1 receptors. Furthermore, OLDA is metabolized (inactivated) slowly, suggesting it is a stable compound that may stay for hours in biomembranes and activate the receptors for longer periods. Moreover, it may function as a central or peripheral mediator of TRPV1 activation.

NADA was the first endovanilloid identified. It has nanomolar potency to TRPV1 and CB1, and is more potent than capsaicin but less so than OLDA. NADA is found in brain regions with high TRPV1 expression (i.e., DRG, hippocampus, striatum and cerebellum). The TRPV1 antagonists, capsazepine and iodoresiniferatoxin, block the action of NADA (Chu et al., 2003, Zhong and Wang, 2008, Hu et al., 2009).

1.5.2 Plant Derived Vanilloids

There are many capsaicinoid analogs found naturally (Kozukue et al., 2005, Thompson et al., 2005a, Thompson et al., 2005b). The six most abundant analogs are DHC, capsaicin, homocapsaicin, homodihydrocapsaicin, nordihydrocapsaicin, and nonivamide (Figure 1-5) (Reilly et al., 2001a, Reilly et al., 2001b, Davis et al., 2007,

Mueller-Seitz et al., 2008). It has been well described that capsaicinoids are important agonists of TRPV1 (Caterina et al., 1999, Zygmunt et al., 1999, Bhawe et al., 2002), and they may produce their effects through both receptor-dependent and receptor-independent pathways (Ziglioli et al., 2009).

Each analog binds to and activates TRPV1, but with different potency depends upon alkyl chain structure (Hayes et al., 1984, Walpole et al., 1993a, Walpole et al., 1993c, Walpole et al., 1993b) and a 3-methoxy-4-hydroxybenzylamine (vanilloid) ring. The most pungent and potent analogs are capsaicin > nonivamide > DHC, followed by the other analogs (Reilly and Yost, 2006).

Capsaicinoids are formed by condensing a branched fatty acid (from 9 to 11 carbon atoms) produced from either leucine or valine, to a molecule of vanillyllamine (Curry et al., 1999, Thiele et al., 2008) and are stable in nonpolar and polar solvents (Tanaka et al., 2009). It has been shown that capsaicinoids exert numerous beneficial physiological and pharmacological effects (Luo et al., 2011). However, capsaicinoids are also toxic to numerous cells through TRPV1-dependent and independent pathways with toxicity dependent on the route of administration (Glinsukon et al., 1980, Lee et al., 2000, Maccarrone et al., 2000, Macho et al., 2000, Surh, 2002, Reilly et al., 2003, Agopyan et al., 2004, Reilly and Yost, 2005, Johansen et al., 2006).

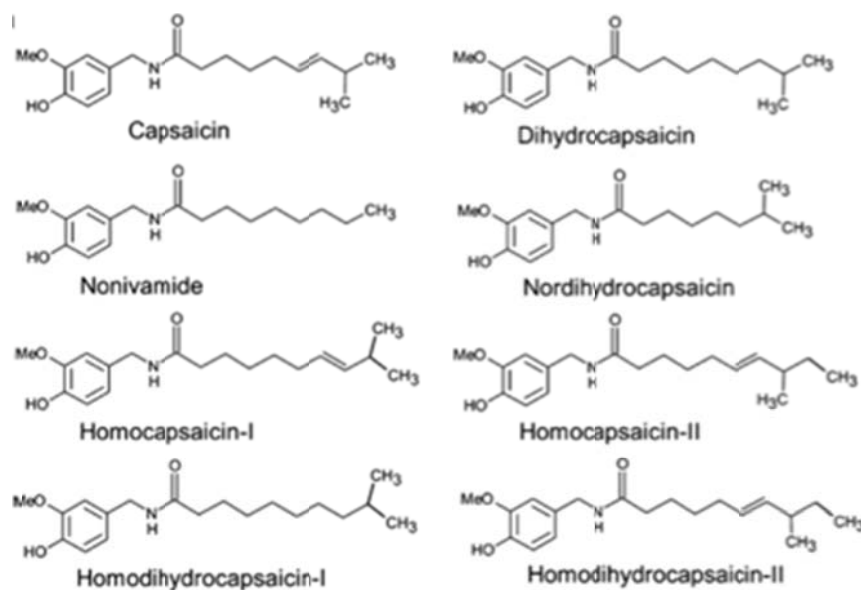


Figure 1-5 Chemical Structures of the Capsaicinoids (Aza-Gonzalez et al., 2010)

1.5.2.1 Plant-Derived Vanilloid Targets and Actions

It is well known that capsaicinoids exert their actions by stimulating TRPV1, however, the exact mechanism(s) for this interaction is still poorly understood (Luo et al., 2011). In the nervous system, capsaicinoids act as natural irritants, selectively activating TRPV1 in sensory neurons that convey noxious sensation to the central nervous system. Activated TRPV1 allows sodium and calcium ions to move through the sensory neuron membrane resulting in depolarization and nociceptive response. This response is followed by a long lasting refractory period and desensitization of the nerve (Wang and Woolf, 2005, Gerner et al., 2008, Kissin, 2008, Knotkova et al., 2008).

TRPV1 can be detected in platelets using Western blotting, and capsaicin stimulates Ca^{2+} entry, or Ca^{2+} release from platelet intracellular stores, and capsaicin also increases platelet cytosolic Na^+ . Those reactions were inhibited by the TRPV1

antagonists, 5-iodo-resiniferatoxin (5-iodo-RTX) and AMG 9810 in a concentration dependent manner. It was also reported that TRPV1 contributed to the activation of platelets by ADP and thrombin which presumably is due to formation of endovanilloids in response to the agonists (Harper et al., 2009).

1.5.2.2 Clinical Applications of Plant-Derived Vanilloids

Capsaicinoids have a number of therapeutic properties but have to date been limited by high toxicity and low selectivity (Luo et al., 2011). Examples include, antineoplastic action (Macho et al., 2003, Yang et al., 2010), pain relief (Wong and Gavva, 2009, Knotkova et al., 2008), anti-obesity, antiinflammatory and antioxidant properties (Ramirez-Romero et al., 2000, Rosa et al., 2002, Sancho et al., 2002, Joo et al., 2010).

1.5.2.2.1 Analgesia

Capsaicin is the most studied capsaicinoid for pain relief. It has been found that administration of capsaicin locally or orally leads to reduced rheumatoid arthritis pain, inflammatory heat and noxious chemical hyperalgesia (Fraenkel et al., 2004). Furthermore, capsaicin is the main component in Adlea, which is a long-acting analgesic ointment used to treat osteoarthritis and post-surgical pain (Remadevi and Szallisi, 2008). Capsaicin is also added to many popular over-the counter creams as a pain reliever, at a concentration of 0.075% or less (Knotkova et al., 2008). Generally, the efficacy of capsaicin-containing creams to treat chronic pain is poor to moderate (Luo et al., 2011). Capsaicin is thought to exert pain relief by an initial irritation and excitation of sensory neurons with TRPV1 receptors, followed by the lasting

refractory state i.e. desensitization (Gerner et al., 2008, Kissin, 2008). That effect makes capsaicinoids unique compare to other natural irritants.

1.5.2.2.2 Cancer Treatment

The potential of capsaicin and DHC in treating cancer has been widely investigated in both *in vitro* and *in vivo* studies (Surh, 2002). In animal experiments, capsaicin reduced the size of MDAMB231 breast cancer masses in mice after oral administration by 50%, and effectively suppressed the growth of breast pre-neoplastic lesions by up to 80%. In addition, systemic administration of capsaicin decreased MDAMB231 breast cancer tumors size by 80% (Thoennissen et al., 2010). Furthermore, capsaicin was able to arrest migration of the cultured breast cancer cell and destroy cultured prostate cancer cells, and DHC was reported to enhance the autophagy in cultured human HCT116 colon cancer cells (Oh et al., 2008, Thoennissen et al., 2010, Yang et al., 2010). Moreover, in clinical studies capsaicin was reported to suppress the development of leukemic cells expressing wild-type p53 (Ito et al., 2004).

It is well known that proliferation of cells plays the main role in carcinogenesis and is an important marker for cancer prevention. The chemopreventive and anticancer effects of capsaicinoids are closely linked to their potential to inhibit cell proliferation and migration, and to induce apoptosis. Moreover, capsaicin and DHC have been found to suppress the development of different malignant cell lines by inducing apoptosis, cycle arrest, autophagy, and/or by the suppression of metabolic cellular activation (Zhang et al., 2003, Choi et al., 2010b, Choi et al., 2010a, Ghosh and Basu, 2010, Thoennissen et al., 2010). It has been reported that capsaicin and

DHC might suppress an isoform of cytochrome P450, which is an enzyme associated with metabolic activation and detoxification of variant low-molecular-weight carcinogens (Singh et al., 2001). Interestingly, capsaicin selectively induces apoptosis or inhibits the growth of malignant or immortalized cell lines, while at the same dosage does not affect normal cell lines (Kim and Moon, 2004). The underlying mechanisms of this phenomenon are still poorly understood (Luo et al., 2011).

In contrast, chilli extracts or capsaicin may instead act as a tumor promoter or co-carcinogen (Surh and Lee, 1996). Epidemiological studies have found that people who consumed chilli peppers in large quantities are at higher risk of stomach cancer than non-consumers. Moreover, capsaicin metabolites (such as the reactive phenoxy radicals) may affect DNA and trigger malignant transformation and mutagenicity (Baez et al., 2010). Thus, capsaicin has both chemopreventive and carcinogenic characteristics, which make it a 'double edged sword' (Luo et al., 2011).

1.5.2.2.3 Weight Reduction

It is well known that energy expenditure and thermogenesis play a major role in obesity regulation, and that chilli peppers are able to increase energy expenditure to produce a sensation of heat when eaten. Therefore, capsaicinoids are considered as a potential natural substance for obesity management (Cui and Himms-Hagen, 1992, Leung, 2008, Joo et al., 2010). In clinical studies and animal experiments it has been found that capsaicinoids suppress obesity by decreasing body fat accumulation (Shin and Moritani, 2007, Reinbach et al., 2009).

1.5.2.2.4 Gastrointestinal System

It is well known that the gastrointestinal system is rich in capsaicin-sensitive sensory nerves, which are thought to play an important role in maintaining the integrity of the gastrointestinal mucosa against injury (Peng and Li, 2010). It has also been reported that capsaicinoids have a gastroprotective effects in various animal models with gastric ulcers induced by hydrochloric acid, aspirin, ammonia, indomethacin or ethanol (Szolcsanyi and Bartho, 2001, Mozsik et al., 2007). This effect is attenuated in the presence of a TRPV1 antagonist (Luo et al., 2011). Conversely, it has been demonstrated that the effects of capsaicinoids on gastrointestinal mucosa are either beneficial or harmful depending on the dose and/or duration of exposure (Wang et al., 2005). Low doses of capsaicinoids may stimulate gastric epithelial regeneration and augment the basal gastric mucosal blood flow and gastric mucus secretion, which are advantageous to gastrointestinal defense (Nishihara et al., 2002). However, high doses of capsaicinoids usually result in depletion of neurotransmitters and desensitization and damage of capsaicin-sensitive neurons, which may injure the gastrointestinal mucosa (Wang, Hu et al. 2005).

1.5.2.2.5 Cardiovascular System

There is growing evidence that capsaicinoids have many beneficial effects on the cardiovascular system (Harada and Okajima, 2009, Peng and Li, 2010). Capsaicin-sensitive sensory neurons are present in the cardiovascular system and may play an important role in controlling cardiovascular function via release of neurotransmitters such as substance P and the vasorelaxant peptide (calcitonin gene-related peptide, CGRP) (Zvara et al., 2006, Peng and Li, 2010). In addition, studies have reported that capsaicin activates TRPV1 to stimulate the release of CGRP, providing a

protective effect on cardiovascular function (Peng and Li, 2010). However, high dose of capsaicin can have deleterious effects on the cardiovascular system due to depletion of CGRP from nerve endings (Peng et al., 2002a, Peng et al., 2002b). The antioxidant property of capsaicinoids also contributes to their beneficial effects on the cardiovascular system. It is believed that low-density lipoprotein (LDL) oxidation is the initiating factor for growth and progression of atherosclerotic plaques. It has been found *in vitro* that capsaicin and DHC were able to raise the LDL resistance to oxidation by slowing the initiation of oxidation and/or delaying the oxidation rate (Ahuja et al., 2006). In high fat-fed rats, it has been reported that serum total cholesterol and lipid peroxide levels are reduced following capsaicin treatment (Manjunatha and Srinivasan, 2006, Manjunatha and Srinivasan, 2007). In adult women and men, the resistance of serum lipoproteins to oxidation increased after four weeks of regular consumption of chilli (Ahuja and Ball, 2006). These findings reflect the antioxidant ability of capsaicinoids and their potential clinical usefulness on cardiovascular diseases prevention (Luo et al., 2011).

1.5.2.2.6 Effects of Plant-Derived Vanilloids on Platelet Aggregation

Recently, it has been reported that capsaicin inhibits *in vitro* platelet aggregation in a concentration-dependent manner, while DHC exhibits a bimodal effect on platelet aggregation, stimulating at 3.125 μM , and inhibiting from 25-100 μM (Adams et al., 2009). Furthermore, it was shown that capsaicin inhibited platelet aggregation induced by different agonists (ADP, calcium ionophore and collagen) but to a lesser extend compared to AA (Raghavendra and Naidu 2009). The anti-haemostatic effects of capsaicinoids may potentially prevent and/or decrease the incidence of

cardiovascular disease risk. The exact mechanism(s) responsible for the effect of capsaicin and DHC on platelet aggregation are not well understood.

Previous studies suggested that the anti-haemostatic effect of capsaicin and DHC was not through specific receptors (TRPV1) on the platelet, but through insertion into platelet plasma membrane and changing membrane ionic permeability and/or fluidity (Hogaboam and Wallace, 1991). A subsequent study reported that capsaicin inhibited platelet aggregation induced by AA in a concentration-dependent manner through inhibition of COX1 (Raghavendra and Naidu 2009). According to Adams *et al.*, (2009), inhibition of platelet aggregation by capsaicin and DHC is unlikely to be mediated through interference with ADP receptors, because the platelet's shape did not change following exposure to capsaicin or DHC. However, a recent study has reported that TRPV1 was present in human platelets, and capsaicin was able to induce a concentration-dependant raise in Ca^{2+} release and Ca^{2+} influx from intracellular platelet stores, which was suppressed by 5-iodo-resiniferatoxin and AMG 9810, the TRPV1 antagonists (Harper et al., 2009). Furthermore, TRPV1 was reported to contribute to the activation of platelets induced by ADP and thrombin, suggesting formation of endovanilloids in response to the platelet agonist (Harper et al., 2009). This finding is in conflict with other recent and earlier studies, which demonstrated that capsaicinoids inhibit platelet aggregation rather than activate platelet aggregation (Adams et al., 2009). A recent study on canine platelets reported that capsaicin inhibits platelet aggregation induced by collagen in a concentration dependent manner. They used a TRPV1 antagonist (A-993610) and found that capsaicin inhibitory effects on platelet aggregation were not mediated via TRPV1 (Mittelstadt et al., 2012).

1.5.2.3 Plant-Derived Vanilloid Toxicity

Vanilloids have been shown to be lethal via all routes of exposure in animals with intravenous administration the most toxic (Glinsukon et al., 1980). They have been widely known for a long time to cause coughing, respiratory inflammation, and severe irritation in experimental human and animals models. Capsaicin-induced cell death was greater in the cells that expressed more TRPV1, suggesting that TRPV1 might be a key mediator of capsaicin-cytotoxic effects in these cells (Reilly, Taylor et al. 2003). In addition, there are many hypotheses to describe the mechanism(s) of bronchial epithelial cell death (Kedei et al., 2001, Grant et al., 2002, Reilly et al., 2005). The cytotoxic effects of capsaicinoids in peripheral sensory (A and C-fiber) neurons has been reported (Szallasi and Blumberg, 1999) and used for chronic pain treatment (McMahon et al., 1991).

1.6 Project Aims

The overall aim is to investigate the effects of vanilloid-like agents, including plant-derived vanilloids (capsaicin and DHC) and endogenous vanilloids (OLDA and NADA) on platelet aggregation.

The specific aims are to:

1. Determine the *in vitro* effects of vanilloid-like agents on platelet aggregation induced by different platelet agonists, namely, ADP, collagen and AA.
2. Determine the effects of transient receptor potential vanilloid (TRPV1) antagonist with vanilloids on platelet aggregation induced by ADP.
3. Determine the potential direct toxic effects of vanilloids on platelets.
4. Determine the effects of vanilloids on platelet alpha granule release.

Chapter 2 Materials and Methods

2.1 Ethics

This study was approved by the Human Research Ethics Committee (Tasmanian) Network (Ref No: H0011414). Informed consent was obtained from each subject in accordance with the Declaration of Helsinki of the World Medical Association.

2.2 Materials

Reagent	Supplier	Location
Adenosine diphosphate Arachidonic Acid Collagen	Helena Laboratories	Beaumont, Texas, USA
Capsaicin N-arachidonoyl-dopamine (NADA) N-oleoyldopamine (OLDA) SB452533 (<i>N</i> -(2-Bromophenyl)- <i>N'</i> -[2-[ethyl(3-methylphenyl)amino]ethyl]-urea)	Tocris Bioscience	Bristol, UK
Dihydrocapsaicin	Sigm-Aldrich Pty.Ltd	NSW, Australia
Beta-Thromboglobulin ELISA Kit Platelet Factor 4 ELISA Kit	Uscn Life Science Inc.	Wuhan, China
Serotonin ELISA Kit	Labor Diagnostika Nord GmbH & Co.KG	Nordhorn, Germany
LDH Cytotoxicity Detection Kit	Clontech Laboratories	Mountainview, CA, USA
All other reagents	Sigma-Aldrich Pty Ltd	NSW, Australia

2.2.1 Preparation of Platelet Aggregation Agonists

ADP and AA were dissolved in deionized water and stored in 1M aliquots at -20°C until needed. Aliquots of agonists were thawed and diluted in normal saline (pH 7.1) to produce final concentration of ADP (5 and 10 μ M), AA (300 and 400mg/mL) and collagen (4 and 8 μ g/mL).

2.2.2 Preparation of Vanilloids

Capsaicin, DHC and OLDA were dissolved in 100% ethanol and stored as 0.1M aliquots at -20°C. NADA was stored as 11.37 x 10³ μ M aliquot at -20°C. Aliquots of all vanilloids were diluted as required in normal saline (pH 7.1) to provide final incubation concentrations ranging from 3.125-100 μ M. The TRPV1 antagonist SB452533 was dissolved in 100% ethanol and stored in 10M aliquot at -20°C and diluted to provide final incubation concentrations (1 and 10 μ M).

2.3 Methods

2.3.1 Platelet Aggregation

2.3.1.1 Principle of Light Transmission Aggregometry

Platelet aggregation testing measures the ability of various agonists to platelets to induce *in vitro* activation and platelet-to-platelet activation. Platelet rich plasma (PRP) is stirred in a cuvette at 37°C and the cuvette sits between a light source and a photocell. When an agonist is added the platelets aggregate and absorb less light and so the transmission increases and this is detected by the photocell. There are two

types of agonists, weak agonists such as ADP, and strong agonists such as collagen. Aggregation induced by weak agonists form primary and secondary waves, while strong agonists form only secondary waves. Sometimes, strong agonists act as weak agonists at low concentrations but weak agonists even at high concentrations do not act like strong agonists (White and Jennings, 1999).

2.3.1.2 Sample Collection and Processing

Venous whole blood was collected by venipuncture from forty-eight normal subjects (aged 18-65) with 21-gauge needles and 25-mL syringes. Samples were collected into 3.2% sodium citrate anticoagulant (1:9 ratio of anticoagulant to whole blood). Volunteers avoided aspirin and antiplatelet medications for at least 10 days and dietary chilli for at least 2 days before giving blood samples.

After collection, specimens were centrifuged at 150g for 10 minutes in room temperature to obtain platelet rich plasma (PRP). Then the remaining blood was centrifuged at 2000g for 20 minutes in room temperature to obtain platelet poor plasma (PPP). The platelet count in PRP was determined using a Sysmex 1000i analyzer and adjusted to $250 \times 10^9/\text{L}$ using PPP from the same donor.

2.3.1.3 Effect of Vanilloids on Platelet Aggregation

All experiments were conducted using the four-channel AggRAM platelet aggregation analyzer (Helena Laboratories, Beaumont, USA) at 600rpm and 37°C using PRP adjusted to $250 \times 10^9/\text{L}$ with PPP from the same donor. 225 μL of PPP was placed in a cuvette in order to zero the machine before each sample analyses. Subsequently, 225 μL of PRP was activated with collagen (4 or 8 $\mu\text{g}/\text{mL}$), ADP (5 or

10 μ M) or AA (300 or 400mg/mL) in the presence and absence of capsaicin, DHC, NADA or OLDA (final concentrations (0-100 μ M). The four cuvettes were placed into the corresponding slot in the AggRAM and the corresponding button was pressed and the counting down was started from 10 minutes, after 40 seconds the agonist with/without the vanilloid was added (no incubation). In each analysis zero level of vanilloid was used with the agonist in the first slot and three different concentrations in the others. Finally, data were calculated automatically by the aggregometer software (HemoRAM 1.1.0., Helena Laboratories, Texas, USA), which are maximum percent of aggregation (%MAX, reflects the intensity of aggregation responses) percent of area under the curve (%AUC), slope (rate of aggregation) and lag time per second (reflects the time from adding the agonist until the aggregation started).

2.3.1.4 Effect of SB-452533 on ADP-Induced Platelet Aggregation

The TRPV1 antagonist SB-452533 (1 and 10 μ M), ADP (5 μ M) and capsaicin or OLDA (50 μ M) were added to PRP, after 40 seconds from the start of aggregation (no incubation). %MAX, slope and %AUC were determined by the aggregometer software (HemoRAM 1.1.0., Helena Laboratories, Texas, USA). This experiment was done to investigate if a SB-452533 would have an effect on OLDA and/or capsaicin inhibitory effect on platelet aggregation induced by ADP.

2.3.2 Lactate Dehydrogenase (LDH) Cytotoxicity Assay

2.3.2.1 Test Principle and Method

The assays were performed according to manufacturer's instructions, Briefly, LDH is a stable cytoplasmic enzyme that is present in all cells. When the cell membrane is damaged, LDH is released rapidly into the culture supernatant. The LDH cytotoxicity detection kit (Clontech Laboratories, USA) is a colorimetric assay to quantitate cytolysis/cytotoxicity, and is depend on the measurement of LDH released from damaged cells. Samples and controls are placed to the appropriate flat-bottom 96-well plate wells in triplicate and centrifuged to obtain cell-free culture supernatant. The reaction mixture is added and incubated with the supernatant. The reaction is stopped by the addition of HCL and the color change is measured spectrophotometrically at 490nm or 490nm (reference wavelength 690nm). The LDH concentration in the samples is calculated by subtracting the average background control value from the average absorbance values of controls and samples. Then subtract the low control value from the sample value and divide to the difference of high control and low control.

2.3.3 Measurement of Alpha Granule Release

The release of platelet factor 4 (PF4) and β -thromboglobulin (β -TG) from alpha granules were determined using ELISA.

2.3.3.1 Test Principle and Method

The assays were performed according to manufacturer's instructions, Briefly, ELISA plate (USCN Life Science Inc., Wuhan, China) wells are pre-coated with monoclonal

antibody specific for PF4 or β TG called biotin-conjugated polyclonal antibody. Standards and samples are placed to the appropriate microtiter plate wells, then Avidin conjugated to Horseradish Peroxidase (HRP) is added. A 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution is added to all wells. The wells that contain PF4 or β TG, biotin-conjugated antibody and enzyme-conjugated Avidin show a change in color. The enzyme-substrate reaction is stopped by the addition of a sulphuric acid (stop) solution and the color change is measured spectrophotometrically at 450nm. The PF4 or β TG concentration in the samples is calculated by comparing the optical density (O.D.) of the samples to the standard curve.

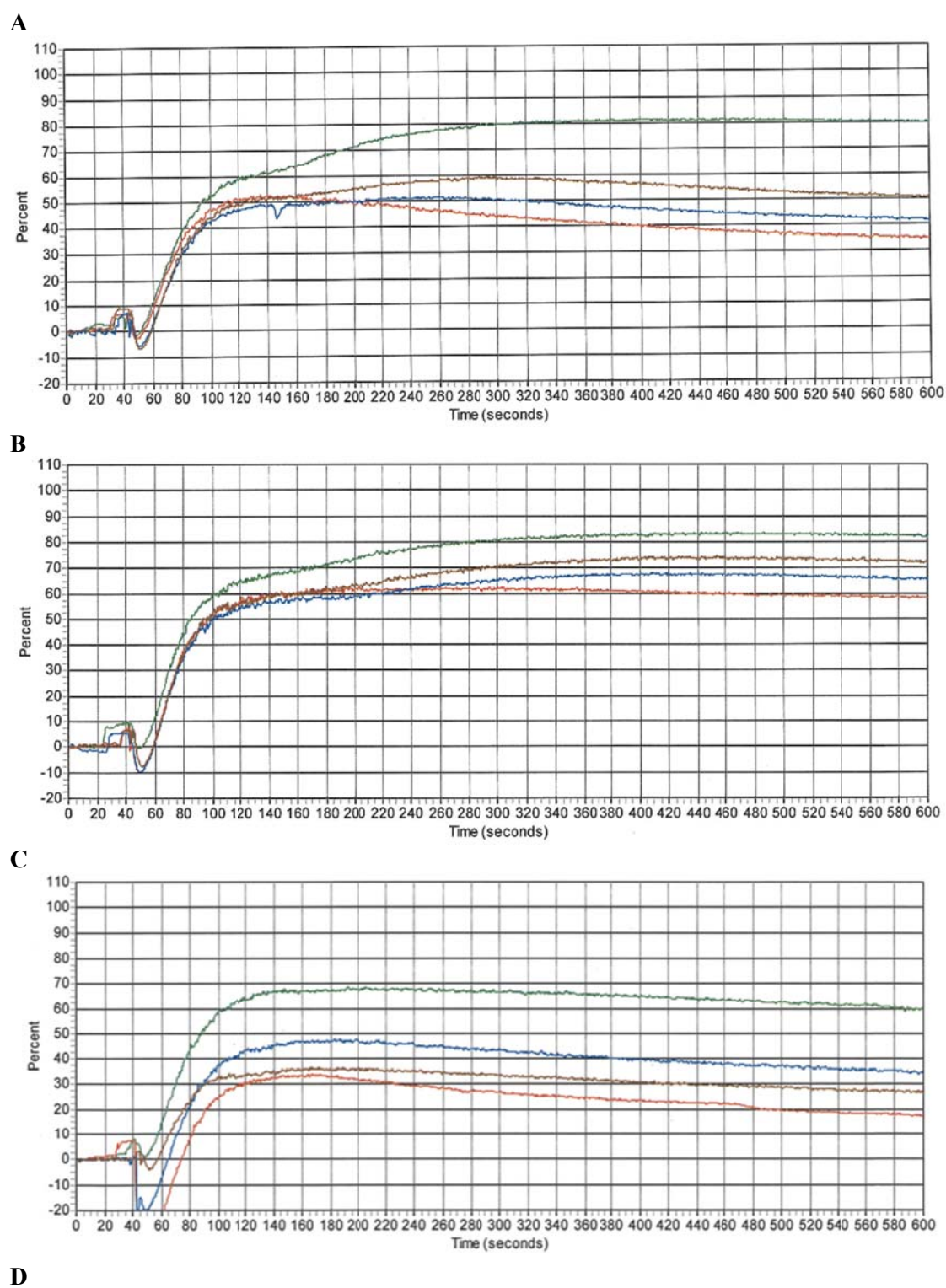
2.3.4 Statistical Analysis

Data were plotted using GraphPad Prism (version 5; San Diego, CA, USA). All data were analysed statistically with repeated measures ANOVA using general linear modelling (STATA version 12SE, StataCorp, LP, USA). Post-estimation Holm test analysis was then used to adjust p values for multiple comparisons. P values < 0.05 were considered significant.

Chapter 3 Results

3.1 Effect of Vanilloid-Like Agents on ADP-Induced Aggregation

All vanilloid-like agents inhibited platelet aggregation induced by ADP. For illustrative purposes, Figure 3.1 demonstrates inhibition of platelet aggregation by these agents.



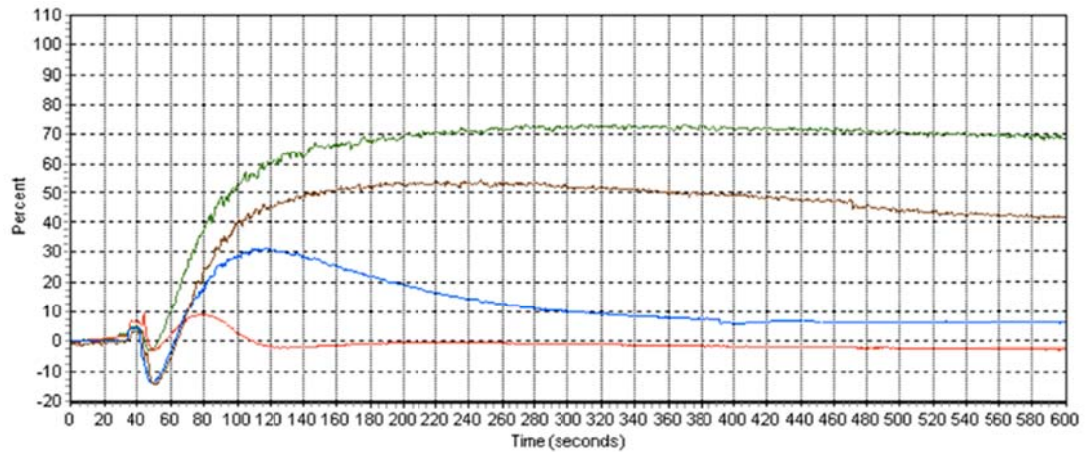


Figure 3-1 Effect of CAP (A), DHC (B), NADA (C) and OLDA (D) on ADP-induced Aggregation. Representative traces showing the effect of vanilloids on platelet aggregation induced by 10µM ADP. The following represents concentrations of vanilloids: green 0µM (saline control); brown 25µM; blue 50µM and red 100µM vanilloid

Concentration-dependent inhibition of ADP (5µM) induced platelet aggregation was observed for capsaicin (0 vs 100 µM; maximum % aggregation (%MAX, 83.8±0.9% vs 45.2±2.4%, $p<0.001$); % area under curve (%AUC, 70.3±0.8 vs 31±3.5, $p<0.001$). Results were similar for 10µM ADP (%MAX, 88±1 vs 69±5.6, $p=0.002$). However, DHC had negligible effect on aggregation induced by 5µM ADP, but inhibited aggregation induced by 10µM ADP in a concentration-dependent manner (%MAX, 73.9±2.8 vs 60.9±4.6, $p=0.007$): (%AUC, 60.2±02.5 vs 47.5±5.1, $p=0.005$) (Figure 3.2 A,B,D,E). Neither capsaicin nor DHC had any effect on the rate of aggregation (slope) (Figure. 3.2 C,F).

The most potent inhibitors of ADP-induced aggregation were OLDA and NADA. OLDA and NADA slightly enhanced aggregation at a concentration of 3.125µM but significantly inhibited it at higher concentrations induced by 5µM and 10µM ADP. Higher concentrations of OLDA suppressed aggregation induced by both 5µM ADP

(%MAX, 71.6 ± 8.2 vs 9.4 ± 1.4 , $p < 0.001$); (%AUC, 58.3 ± 7.2 vs 0.89 ± 0.5 , $p < 0.001$) and by $10 \mu\text{M}$ ADP (%MAX, 84 ± 3 vs 12.6 ± 2 , $p < 0.001$); (%AUC 69.1 ± 2.4 vs 0.81 ± 0.15 , $p < 0.001$). At high concentrations of NADA platelet aggregation induced by $5 \mu\text{M}$ ADP was inhibited (%MAX, 71.5 ± 5.9 vs 38.2 ± 1.4 , $p < 0.008$); (%AUC, 58.8 ± 4.3 vs 26.6 ± 13.7 , $p = 0.003$) and by $10 \mu\text{M}$ ADP (%MAX, 74 ± 4.7 vs 39.2 ± 2.4 , $p < 0.001$); (%AUC, 74 ± 4.7 vs 39.2 ± 2.4 , $p < 0.001$) (Figure 3.3 A,B,D,E). The order of potency in inhibiting ADP-induced platelet aggregation was OLDA > NADA > capsaicin > DHC. The slope of the aggregation curve for both 5 and $10 \mu\text{M}$ ADP was significantly reduced at $100 \mu\text{M}$ OLDA and at $100 \mu\text{M}$ capsaicin for $5 \mu\text{M}$ ADP (Figure 3.2-3.3 C,F). For OLDA, this was most likely an artifact due to the near complete inhibition of aggregation at $100 \mu\text{M}$. The lag time for aggregation was not affected by any of the vanilloid agents tested (data not shown).

Ethanol alone showed no effect on platelet aggregation induced by ADP (data not shown).

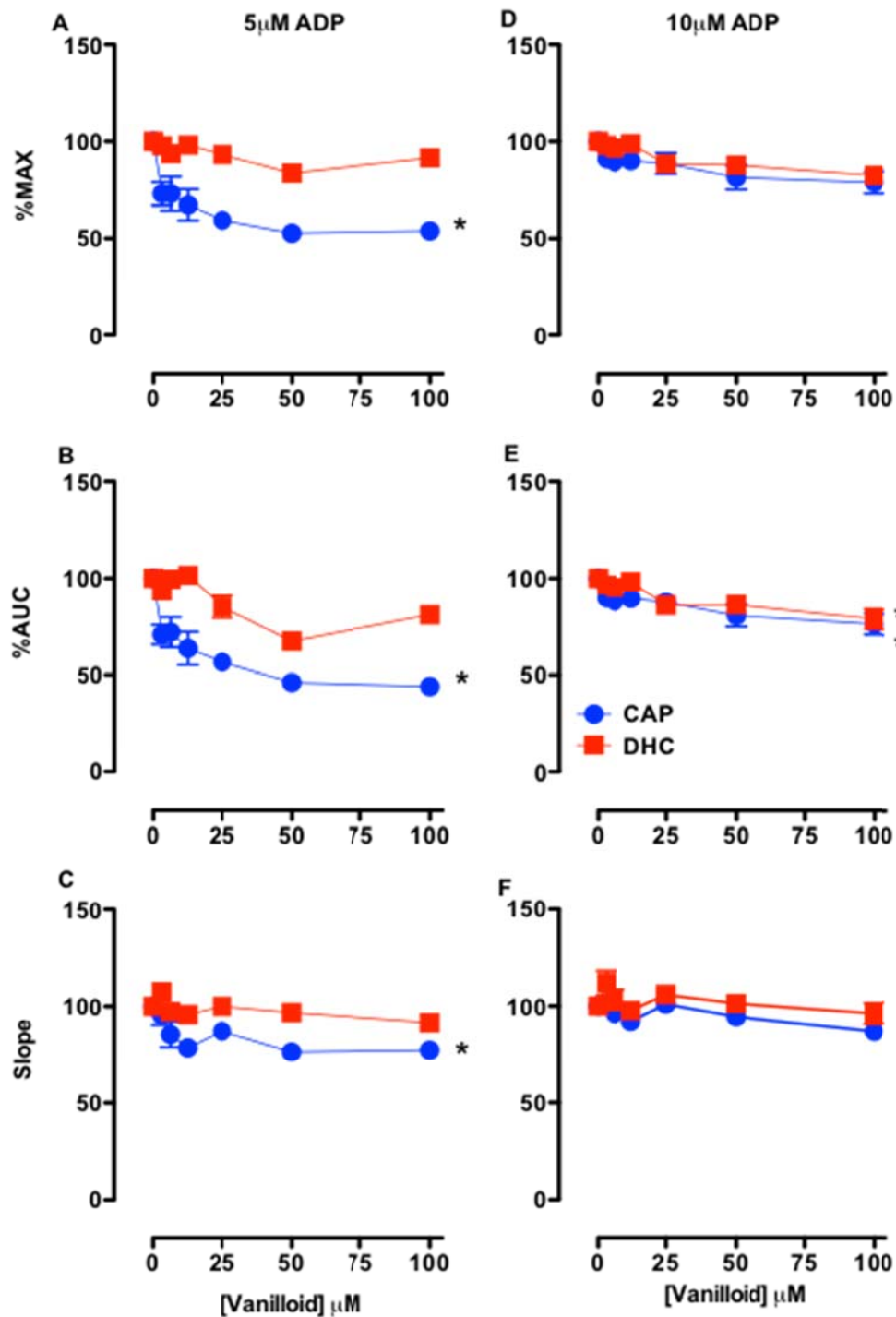


Figure 3-2 Effect of Plant-Derived Vanilloids on ADP-induced Platelet Aggregation. Data are presented as percent maximum aggregation (%Max, normalized to aggregation in the absence of vanilloid) (A,D), percent area under curve (%AUC) (B,E) and slope of the propagation phase of curve (slope) (C,F). Points are the mean \pm SEM of 4 experiments, for capsaicin (CAP) and dihydrocapsaicin (DHC). * $P < 0.05$ compared to control (i.e. 0 μM vanilloids)

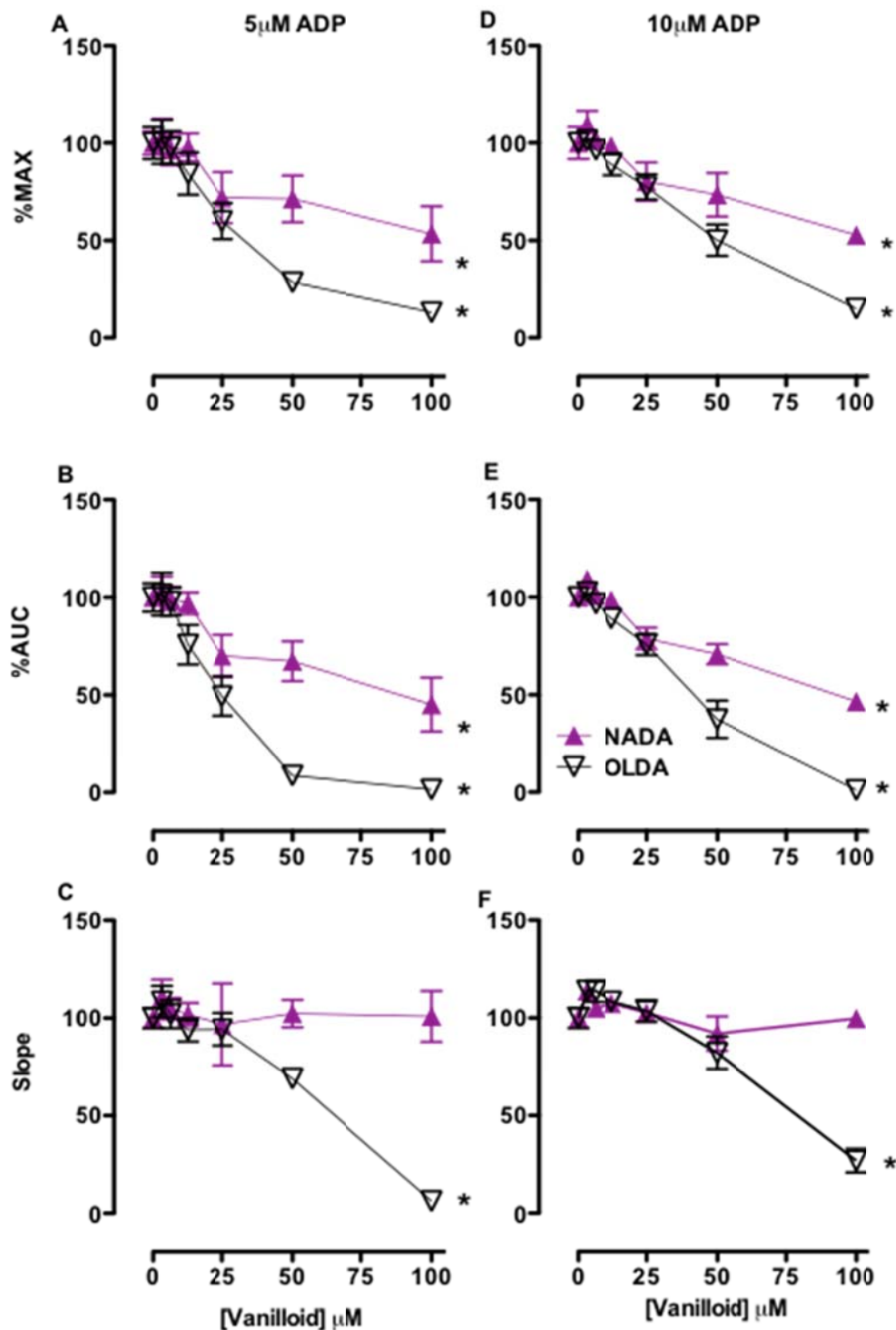


Figure 3-3 Effect of Endovanilloids on ADP-induced Platelet Aggregation.

Data are presented as percent maximum aggregation (%Max, normalized to aggregation in the absence of vanilloid) (A,D), percent area under curve (%AUC) (B,E) and slope of the propagation phase of curve (slope) (C,F). Points are the mean \pm SEM of 4 experiments, for N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA).

* $P < 0.05$ compared to control (i.e. 0 μM vanilloids)

3.2 Effect of Vanilloid-Like Agents on Collagen-Induced Aggregation

OLDA and NADA inhibited platelet aggregation induced by collagen. For illustrative purposes, Figure 3.4 demonstrates inhibition of platelet aggregation by NADA and OLDA.

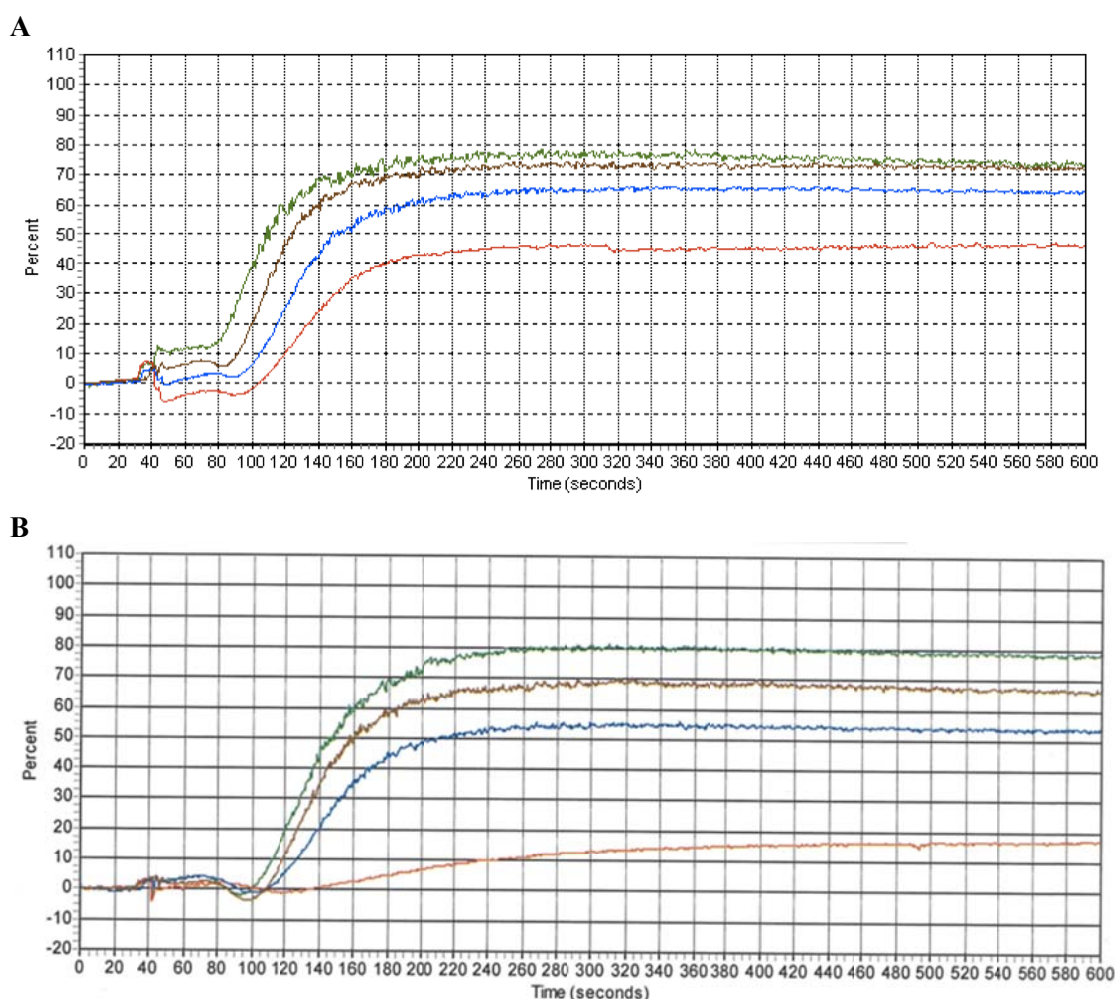


Figure 3-4 Effect of NADA (A) and OLDA (B) on Collagen-induced Aggregation. Representative traces showing the effect of NADA on platelet aggregation induced by 4 μg/mL collagen. The following represents concentrations of NADA: green 0 μM; brown 25 μM; blue 50 μM and red 100 μM vanilloid

Capsaicin and DHC had no effect on any of the measured parameters of platelet aggregation-induced by collagen 4 or 8 μg/mL collagen (Figure 3.5). Platelet aggregation induced by 4 μg/mL collagen but not 8 μg/mL collagen was significantly inhibited by NADA (0 vs 100 μM; %MAX, $87.7 \pm 0.8\%$ vs $28.3 \pm 8.2\%$, $p < 0.001$;

%AUC, 68.8 ± 0.68 vs 19.7 ± 7.3 , $P < 0.001$), and OLDA ($89.3 \pm 1.4\%$ vs $45.5 \pm 12.5\%$, $p < 0.001$; %AUC, 69.9 ± 1.1 vs 32 ± 8.9 , $p < 0.001$) (Figure 3.6 A,B,D,E).

Lag times were non-significantly prolonged with a combination of $4 \mu\text{g/mL}$ collagen and vanilloids especially OLDA ($23 \pm 13.2\text{s}$ vs $74.5 \pm 21.8\text{s}$) and NADA (Lag $26 \pm 12\text{s}$ vs $72.8 \pm 31.2\text{s}$) (Figure 3.7). The slope of the aggregation curve $4 \mu\text{g/mL}$ collagen was significantly reduced at $100 \mu\text{M}$ OLDA and NADA (Figure 3.6 C,F).

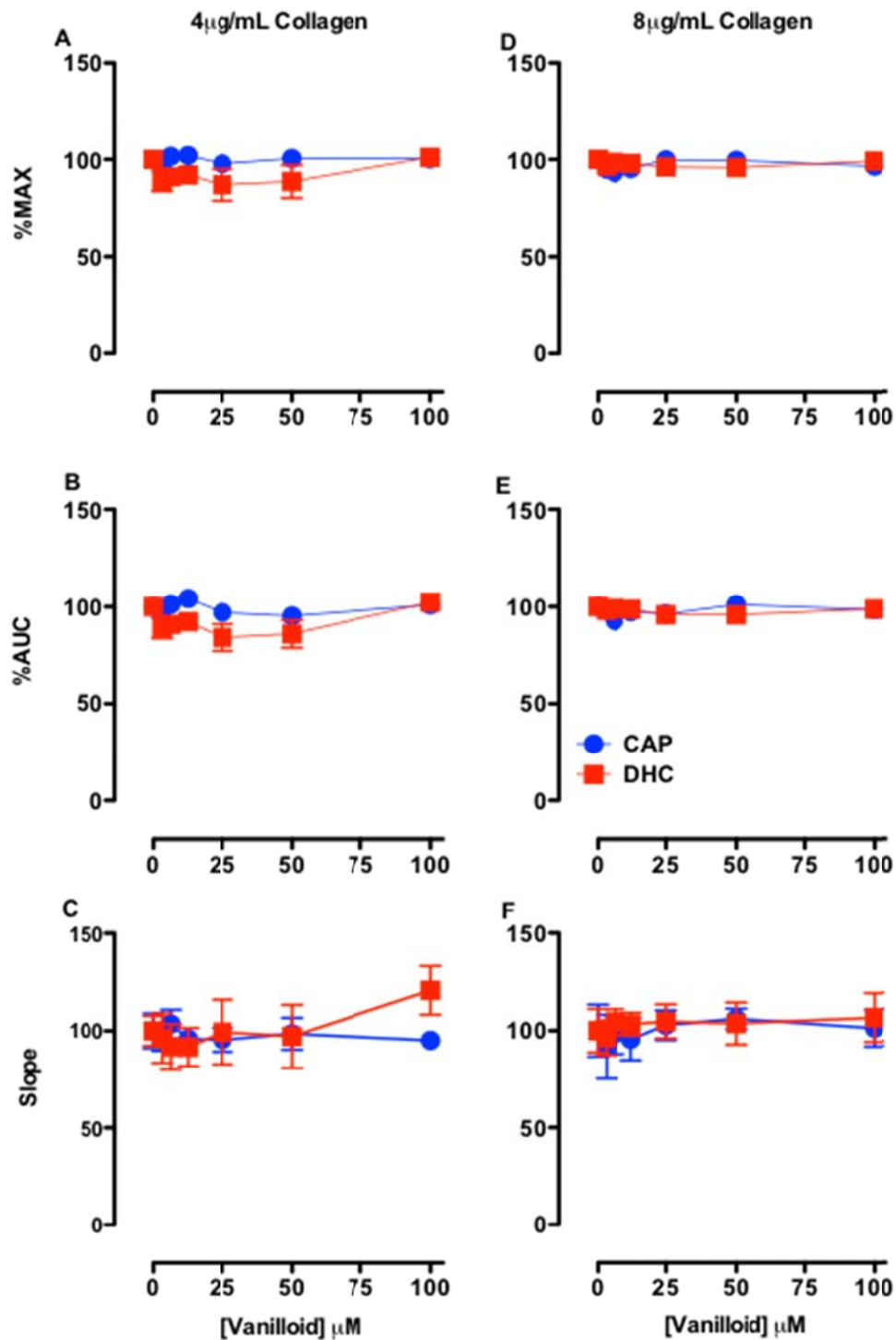


Figure 3-5 Effects of Plant-Derived Vanilloids on Collagen-induced Platelet Aggregation. Data are presented as percent maximum aggregation (%Max, normalized to aggregation in the absence of vanilloid) (A,D), percent area under curve (%AUC) (B,E) and slope of the propagation phase of curve (slope) (C,F). Points are the mean \pm SEM of 4 experiments, for CAP and DHC. * $P < 0.05$ compared to control (i.e. 0 μM vanilloids)

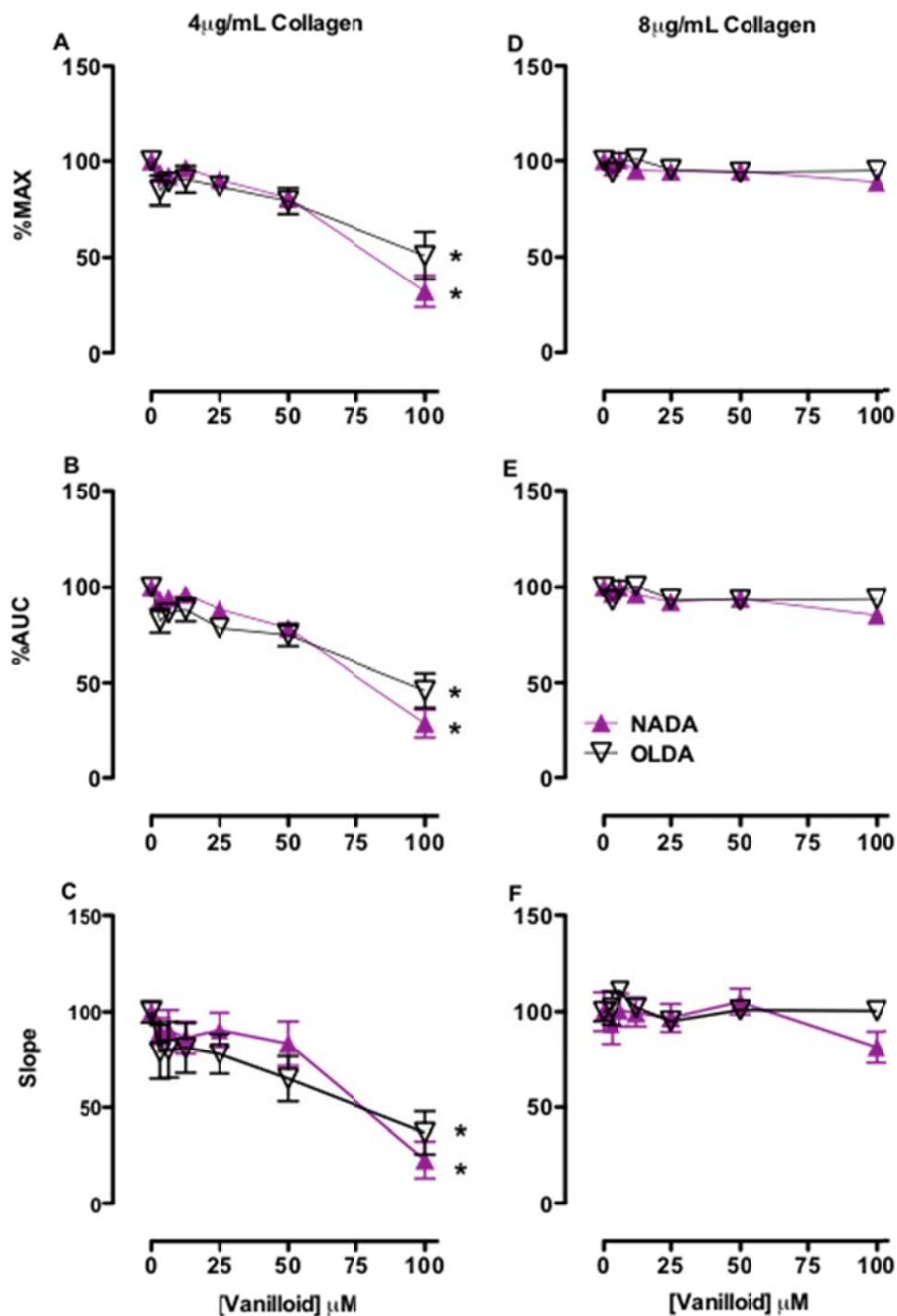


Figure 3-6 Effects of Endovanilloids on Collagen-induced Platelet Aggregation. Data are presented as percent maximum aggregation (%Max, normalized to aggregation in the absence of vanilloid) (A,D), percent area under curve (%AUC) (B,E) and slope of the propagation phase of curve (slope) (C,F). Points are the mean \pm SEM of 4 experiments, for NADA and OLDA. * $P < 0.05$ compared to control (i.e. 0 μM vanilloids)

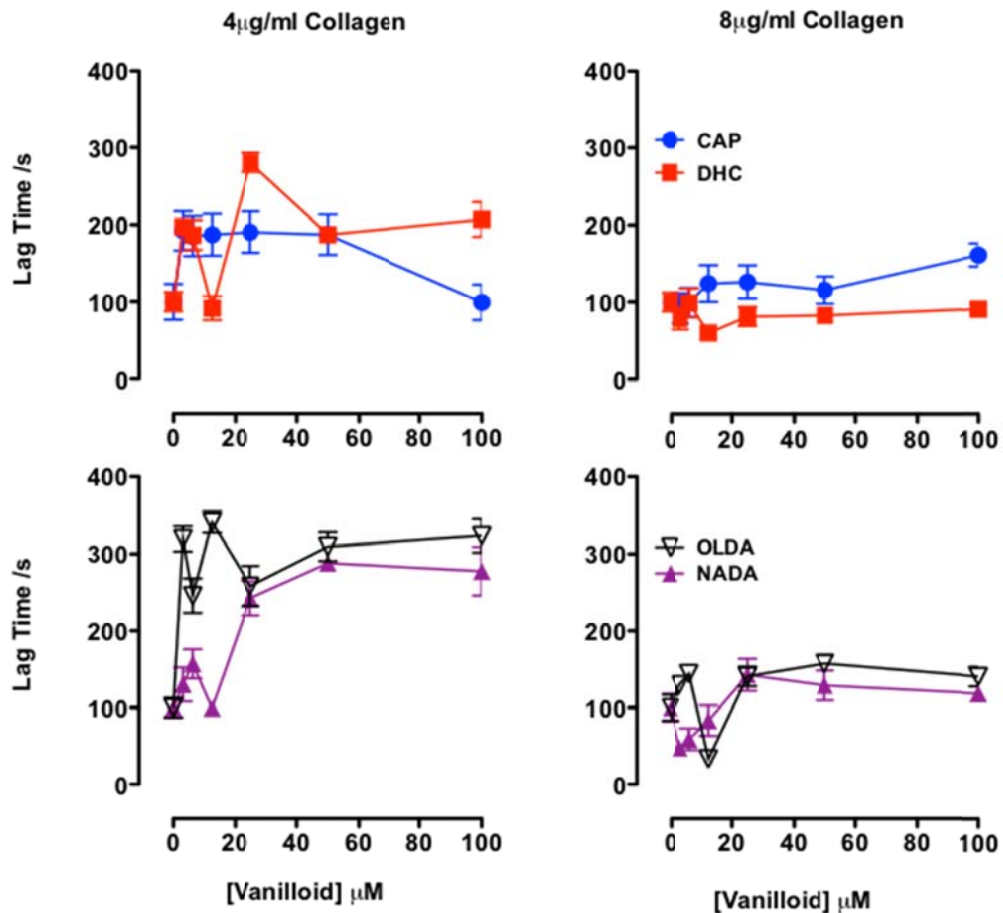


Figure 3-7 Effect of Vanilloids on the Lag-Time of Collagen-induced Platelet Aggregation. Data are presented lag time. Points are the mean \pm SEM of 4 experiments, for CAP, DHC, NADA and OLDA

3.3 Effect of Vanilloid-Like Agents on Arachidonic Acid-Induced Aggregation

Capsaicin, DHC and NADA inhibited platelet aggregation induced by AA. For illustrative purposes, Figure 3.8 demonstrates inhibition of platelet aggregation by capsaicin, DHC and NADA.

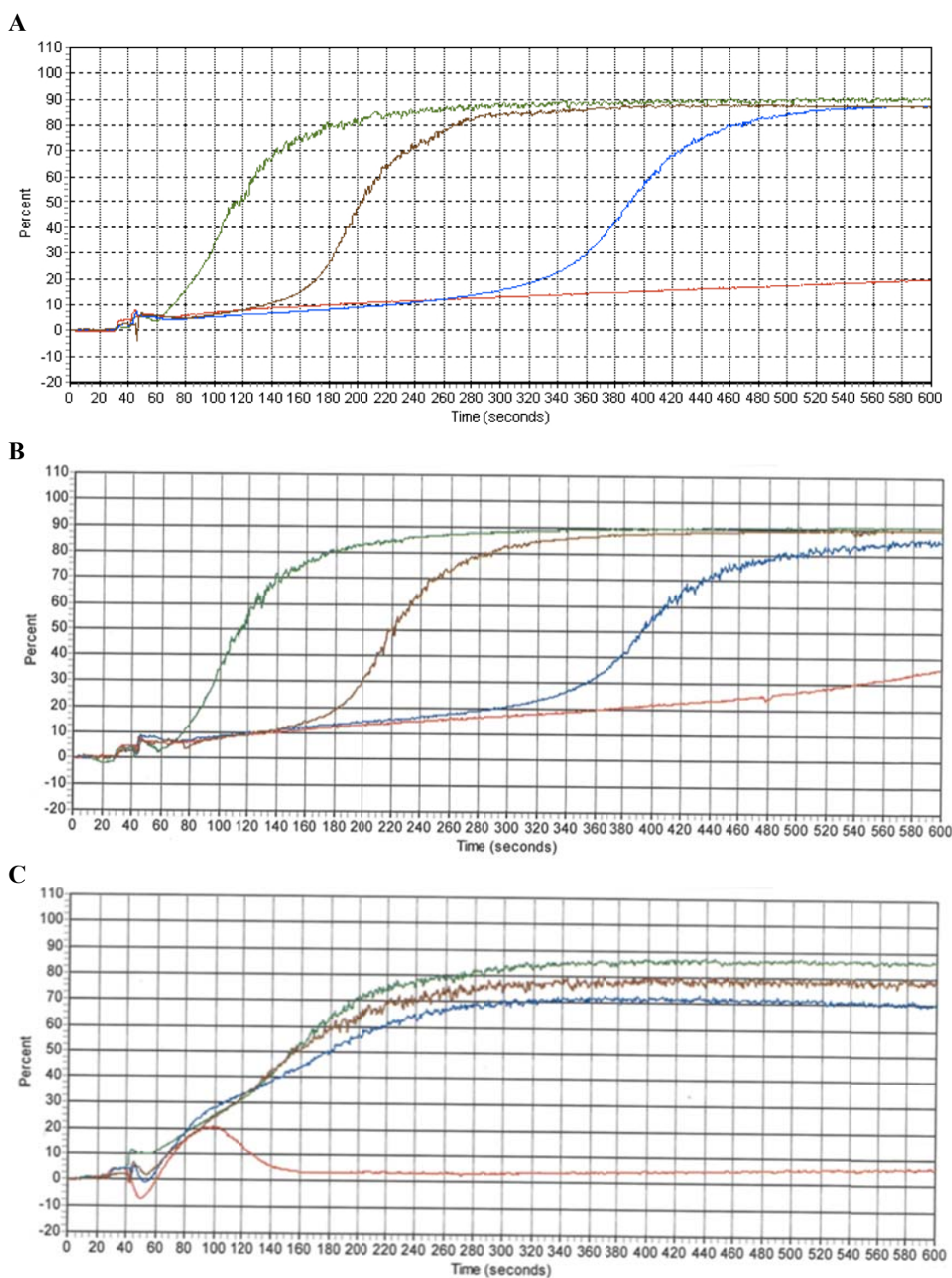


Figure 3-8 Effect of Capsaicin (A), DHC (B) and NADA (C) on Arachidonic Acid-induced Aggregation. Representative traces showing the effect of capsaicin on platelet aggregation induced by 400mg/mL AA. The following represents concentrations of capsaicin: green 0 μ M; brown 25 μ M; blue 50 μ M and red 100 μ M vanilloid

Capsaicin strongly inhibited platelet aggregation induced by 300mg/mL AA (0 vs 100 μ M; %MAX, 89.6 ± 0.9 vs 11 ± 0.8 , $p<0.001$; %AUC, 68.2 ± 2.5 vs 7.34 ± 0.36 , $p<0.001$) and by 400mg/mL AA (91.2 ± 1 vs 27 ± 11.1 , $p<0.001$; %AUC, 68.5 ± 2 vs 11.5 ± 1.9 , $p<0.001$). DHC significantly inhibited aggregation stimulated by 300mg/mL AA (%MAX, 88.3 ± 2.1 vs 18.7 ± 6.9 , $p<0.001$; %AUC, 67.4 ± 3.2 vs 10.2 ± 2.8 , $p<0.001$) and 400mg/mL AA (%MAX, 87.9 ± 3.2 vs 37.8 ± 19.4 , $p=0.037$; %AUC, 65.9 ± 2.6 vs 22.5 ± 8.7 , $p<0.001$). NADA inhibited platelet aggregation induced by 300mg/mL (%MAX, 84 ± 1.8 vs 21.9 ± 4.7 , $p<0.001$; %AUC, 60.3 ± 2 vs 6.9 ± 0.7 , $p<0.001$) and 400mg/mL AA (%MAX, 89.9 ± 2.7 vs 60.4 ± 15.8 , $p<0.002$; %AUC, 65 ± 2.6 vs 44 ± 16 , $p=0.04$) but to a lesser extent compared to the plant-derived vanilloids, capsaicin and DHC. However, capsaicin, DHC and NADA inhibited aggregation induced by 400mg/mL AA in inconsistent manner (Figure 3.9-3.10 A,B,D,E).

The slope of the aggregation curve for both 300 and 400mg/mL AA was significantly reduced at 100 μ M capsaicin and DHC (Figure 3.9 C,F). Lag times were significantly decreased with a combination of 300mg/mL AA and plant-derived vanilloids capsaicin (33.5 ± 9.7 s vs 10.8 ± 7.2 s) and DHC (23.4 ± 13.7 s vs 10.9 ± 4.3 s). In addition, lag time was significantly prolonged with a combination of 400mg/mL AA and NADA (8.3 ± 3.9 s vs 39.2 ± 15.2 s) (Figure 3.11). Finally, at the highest concentration tested (100 μ M), OLDA did not affect platelet aggregation induced by either 300 or 400mg/mL AA (Figure 3.10).

Table 3-1 summarizes the effects of plant-derived vanilloids and endovanilloids on platelet aggregation induced by ADP, collagen and AA.

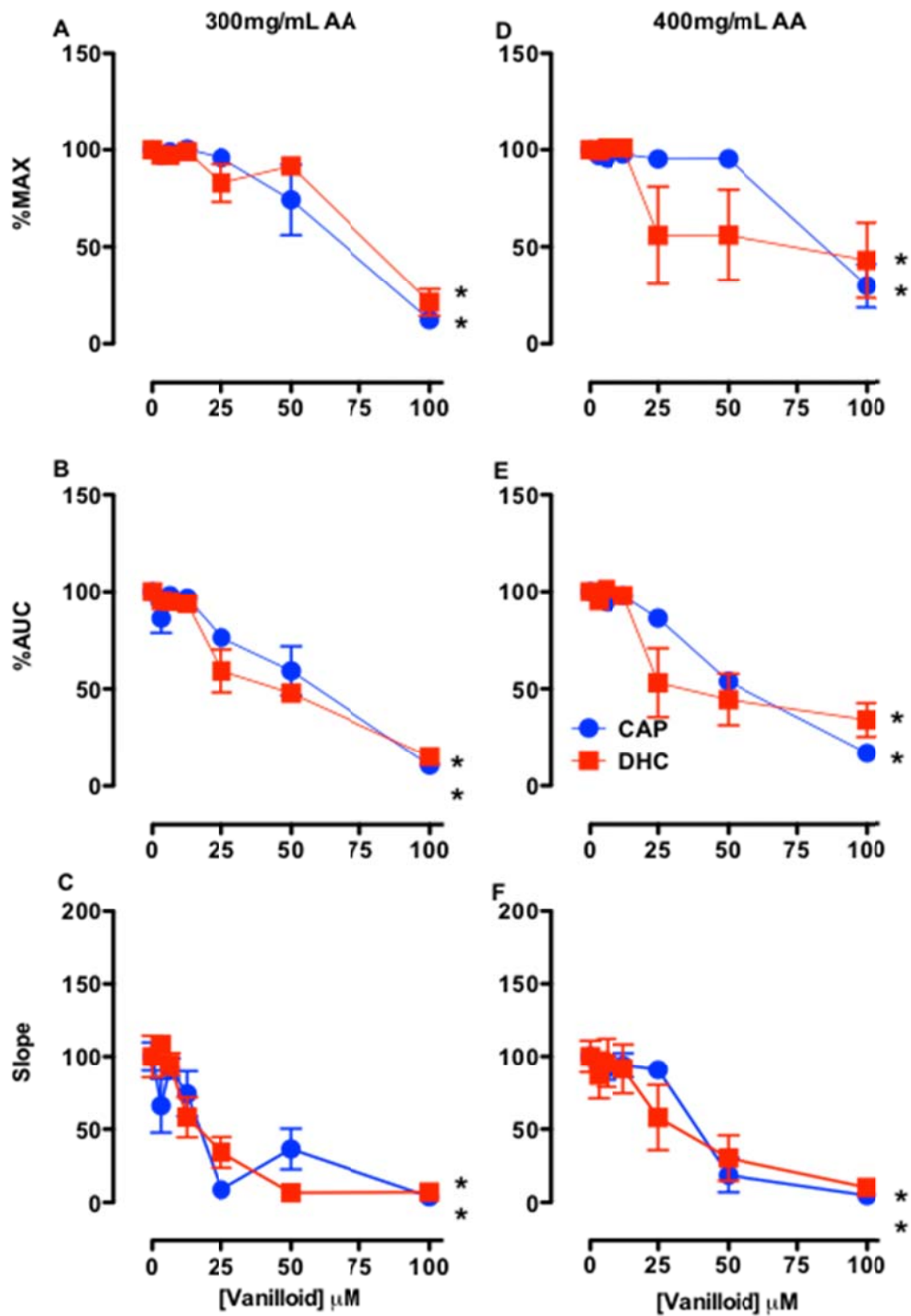


Figure 3-9 Effects of Plant-Derived Vanilloids on Arachidonic Acid-induced Platelet Aggregation. Data are presented as percent maximum aggregation (%Max, normalized to aggregation in the absence of vanilloid) (A,D), percent area under curve (%AUC) (B,E) and slope of the propagation phase of curve (slope) (C,F). Points are the mean \pm SEM of 4 experiments, for CAP and DHC. * $P < 0.05$ compared to control (i.e. 0 μM vanilloids)

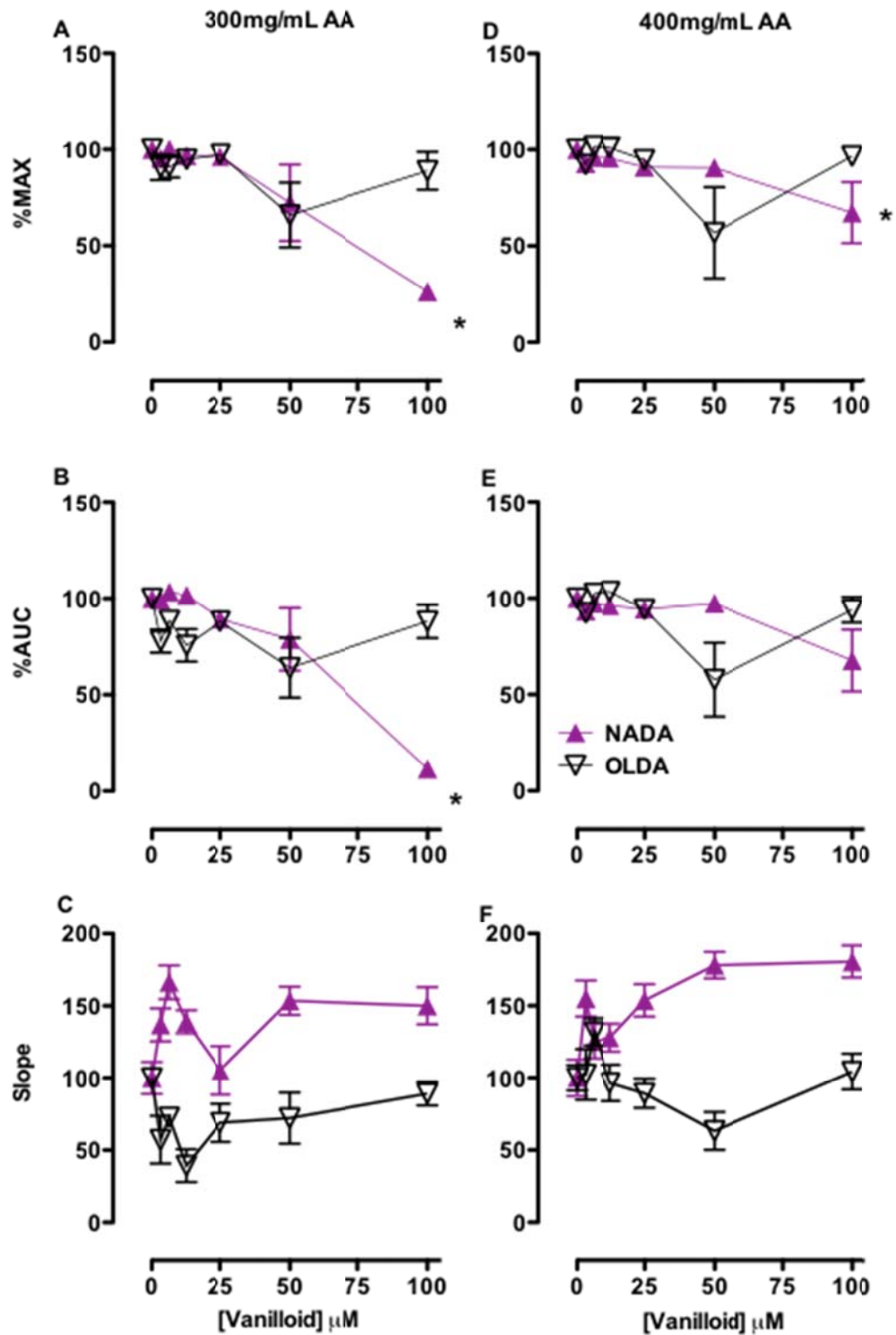


Figure 3-10 Effects of Endovanilloids on Arachidonic Acid-induced Platelet Aggregation. Data are presented as percent maximum aggregation (%Max, normalized to aggregation in the absence of vanilloid) (A,D), percent area under curve (%AUC) (B,E) and slope of the propagation phase of curve (slope) (C,F). Points are the mean \pm SEM of 4 experiments, for NADA and OLDA. * $P < 0.05$ compared to control (i.e. 0 μM vanilloids)

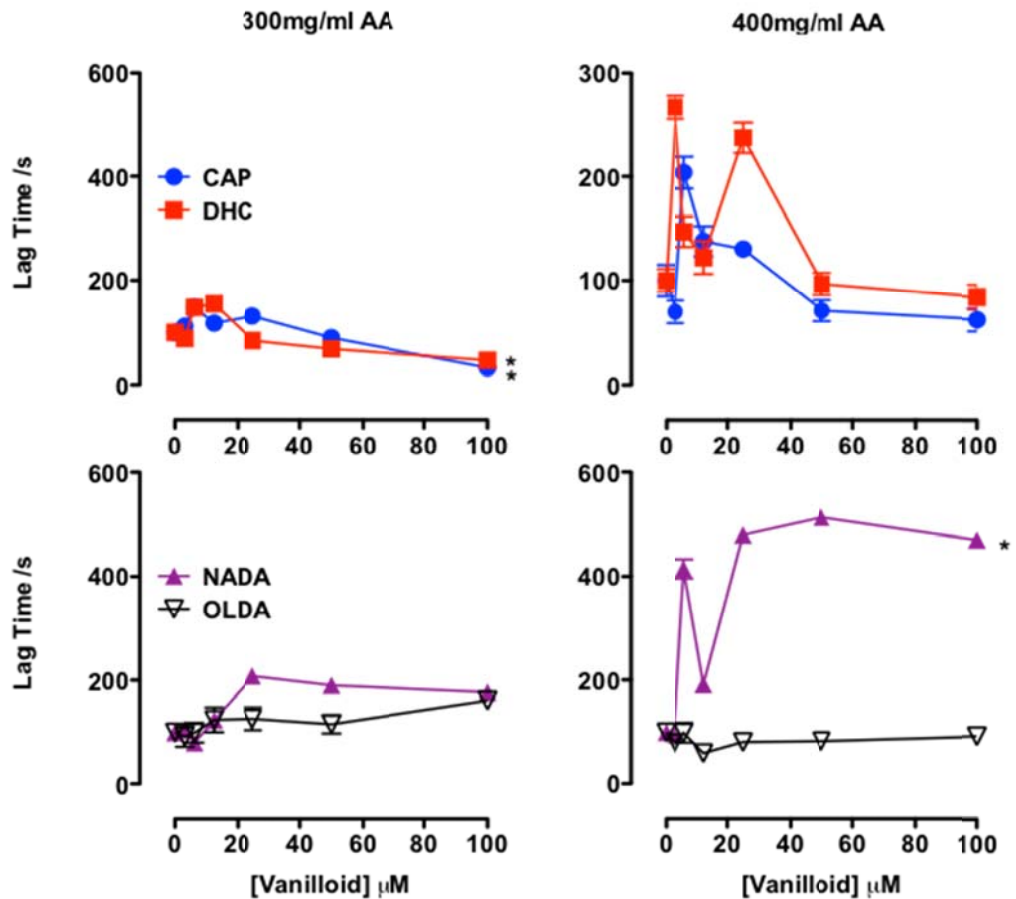


Figure 3-11 Effect of Vanilloids on the Lag-Time of Arachidonic Acid-induced Platelet Aggregation. Data are presented lag time. Points are the mean \pm SEM of 4 experiments, for CAP, DHC, NADA and OLDA. * $P < 0.05$ compared to control (i.e. 0 μM vanilloids)

Table 3-1 Effect of Vanilloids on ADP, Collagen, Arachidonic acid-induced Platelet Aggregation.

Agonist	ADP (5μM)							
	CON	CAP	CON	DHC	CON	NADA	CON	OLDA
% MAX	83.8±0.9	45.2±2.4 ***	78.8±2.8	60.9±9.1	71.5±5.9	38.2±1.4 **	71.6±8.2	9.4±1.4 ***
% AUC	70.3±0.8	31.0±3.5 ***	60.2±2.5	47.5±5.1	58.8±4.3	26.6±13.7 **	58.3±7.2	0.89±0.5 ***
Slope	83.0±1.7	64.2±4.8	84.3±2.1	81.0±5.3	80.3±2.0	81.0±7.5	85.3±5.2	5.3±5.8 ***
Agonist	ADP (10μM)							
	CON	CAP	CON	DHC	CON	NADA	CON	OLDA
% MAX	88.0±1.0	69.0±5.6 **	73.9±2.8	60.9±4.6 **	74.0±4.7	39.2±2.4 ***	84.0±3.0	12.6±2.0 ***
% AUC	73.5±1.1	56.0±5.4 **	60.2±2.5	47.5±5.1 **	74.0±4.7	39.2±2.4 ***	69.1±2.4	0.81±0.1 ***
Slope	90.2±1.1	78.5±2.4	84.3±2.1	81.1±5.3	82.7±6.0	82.5±4.8	86.8±5.0	23.3±6.0 ***
Agonist	Collagen (4μg/mL)							
	CON	CAP	CON	DHC	CON	NADA	CON	OLDA
% MAX	83.9±2.4	84.3±1.0	89.4±1.3	90.4±1.0	87.7±0.8	28.3±8.2 ***	89.3±1.4	45.5±12.5 ***
% AUC	67.0±1.5	67.7±0.7	70.6±1.2	72.3±0.8	68.8±0.68	19.7±7.3 ***	69.9±1.1	32.0±8.9 ***
Slope	104.6±8.9	99.6±3.5	98.2±7.7	118.6±12.5	108.7±4.1	24.7±9.5 ***	111.5±5.6	41.0±11.2 ***
Agonist	Collagen (8μg/mL)							
	CON	CAP	CON	DHC	CON	NADA	CON	OLDA
% MAX	85.3±2.8	82.3±2.8	91.0±1.6	90.4±1.0	88.9±2.6	79.0±2.7	90.3±1.6	85.7±1.7
% AUC	69.6±1.8	68.5±1.9	73.0±2.6	72.3±0.8	72.4±1.8	61.7±3.0	72.0±1.4	67.4±1.9
Slope	114±13.4	115.4±9.4	111.2±11.0	118.6±12.5	120.0±10.0	97.9±8.0	114.0±4.9	114.6±4.3

Table 3-1 (cont). Effect of Vanilloids on ADP, Collagen, Arachidonic acid-induced Platelet Aggregation.

Agonist		AA (300mg/mL)						
CON	CAP	CON	DHC	CON	NADA	CON	OLDA	CON
% MAX	89.6±0.9	11.0±0.8 ***	88.3±2.1	18.7±6.9 ***	84.0±1.8	21.9±4.7 ***	81.5±1.6	72.4±9.8
% AUC	68.2±2.5	7.34±0.36 ***	67.4±3.2	10.2±2.8 ***	60.3±2.0	6.9±0.7 ***	64.3±0.6	56.7±8.6
Slope	74.4±9.7	2.9±0.3 ***	58.4±14.4	4.2±0.6 ***	31.2±11.0	46.9±12.8	61.6±4.0	54.9±8.0
Agonist		AA (400mg/mL)						
% MAX	91.2±1.0	27.0±11.1 ***	87.9±3.2	37.8±19.4 *	89.9±2.7	60.4±15.8 **	86.0±4.0	83.2±0.5
% AUC	68.5±2.0	11.5±1.9 ***	65.9±2.6	22.5±8.7***	65.0±2.6	44.0±16.0 *	64.0±1.4	60.3±6.5
Slope	71.6±4.3	3.4±0.9 ***	62.0±10.8	6.3±2.0 *	36.9±12.6	66.7±11.0	54.9±8.7	57.3±12.3

Data are presented as percent maximum aggregation (%MAX), percent area under curve (%AUC) and slope of the propagation phase of curve (slope). Data are the mean ± SEM of 4 experiments for (100µM) capsaicin (CAP), dihydrocapsaicin (DHC), N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA). * P <0.05, ** P <0.01, *** P <0.001 compared to control (CON) (i.e. 0µM vanilloids).

3.4 Effect of SB-452533 on ADP-induced Platelet Aggregation

To determine whether inhibition of ADP-induced aggregation by vanilloids was mediated by TRPV1, experiments were conducted in the presence of the TRPV1 antagonist, SB-452533. SB-452533 did not affect the inhibition of ADP-induced aggregation by OLDA (%Max, 0 vs 10 μ M, 55.9 \pm 2.1% vs 58.4 \pm 1.37%) or capsaicin (SB-452533; %Max, 0 vs 10 μ M, 65.15 \pm 0.44% vs 65.55 \pm 1%), suggesting that inhibition of platelet aggregation by vanilloids is not TRPV1 mediated (Figure 3.12).

3.5 Cytotoxicity Assay

LDH release from platelets was not affected when exposed to ADP and/or capsaicin, DHC, OLDA and NADA up to 100 μ M. Hence, at the highest concentration tested in platelet aggregation studies, vanilloids were not toxic to platelets (Table 3-2).

Table 3-2 Cytotoxic effects of vanilloids and endovanilloids on platelets.

Vanilloids	0μM	25μM	100μM
CAP	0%	0%	0%
DHC	0%	0%	0%
NADA	0%	0%	0%
OLDA	0%	0%	0%

Data are presented as percent (%) of cytotoxicity of 4 experiments for capsaicin (CAP), dihydrocapsaicin (DHC), N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA). Data are the percent of triplicate absorbance value – low control, divided by high control – low control.

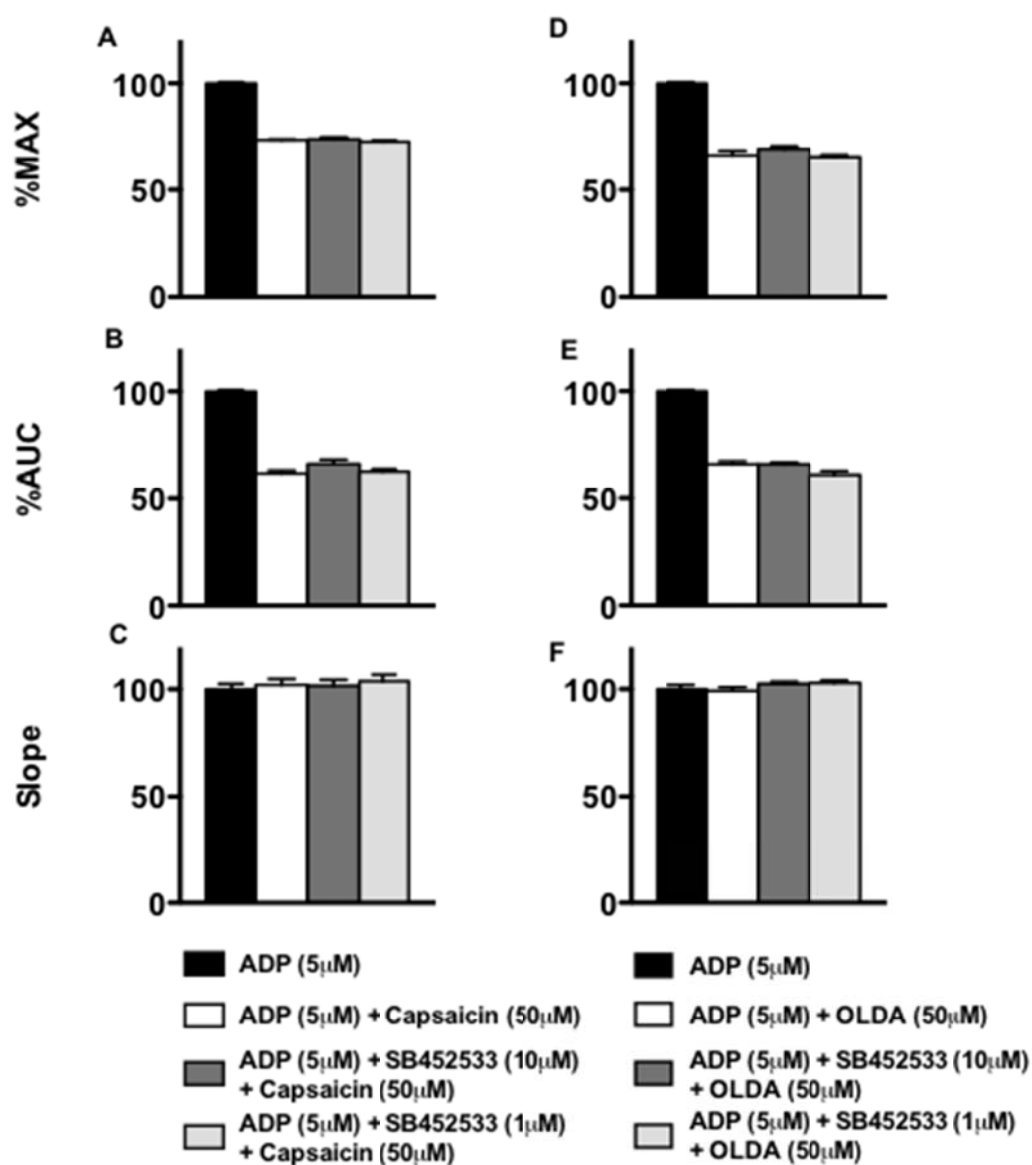


Figure 3-12 Effects of SB-452533 on Capsaicin- and OLDA-mediated inhibition of Platelet Aggregation induced by ADP. Data are presented as percent maximum aggregation (%Max, normalized to aggregation in the absence of vanilloid) (A,D), percent area under curve (%AUC) (B,E) and slope of the propagation phase of curve (slope) (C,F). Points are the mean \pm SEM of 4 experiments, for CAP and OLDA

3.6 Effect of Vanilloids on Alpha Granule Release

Capsaicin, DHC and OLDA inhibited the release of PF4 in a concentration-dependent manner by 5 and 10 μ M ADP. NADA (3.125 and 25 μ M) enhanced PF4 release, whereas 100 μ M NADA inhibited PF4 release (Figure 3.13 C,D).

In the presence of 5 μ M ADP, all vanilloids increased β -TG release, while at 10 μ M ADP, only DHC and NADA increased release. However, capsaicin inhibited β -TG release in a concentration-dependent manner. β -TG release was inhibited by 3.125 and 25 μ M OLDA inhibited at then evoked at 100 μ M. These results are for two samples only and need to be validated (Figure 3.13 A,B).

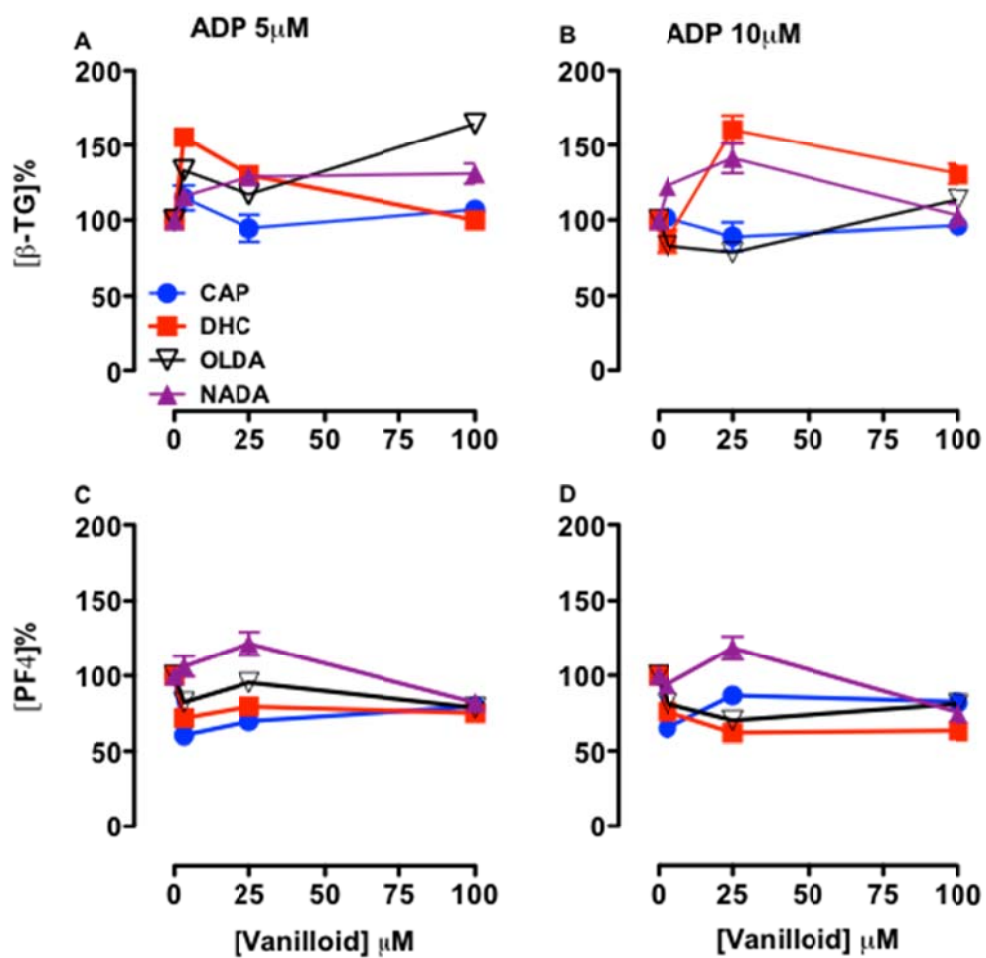


Figure 3-13 Effects of Vanilloids on Platelet Alpha Granule Release. Data are presented as concentration percent as control β -thromboglobulin (A,B) and platelet factor 4 (C,D). Points are the mean \pm SD of 2 experiments, for CAP, DHC, NADA and OLDA

Chapter 4 Discussion

Platelets play an important role in thrombogenesis, with antiplatelet medications such as the cyclooxygenase inhibitors, the most effective drugs for the treatment and prevention of atherothrombotic diseases (Patrono et al., 2004). These drugs however, have a number of side effects such as gastrointestinal bleeding and urticaria (Group, 1991, Mastalerz et al., 2004). Naturally occurring antiplatelet compounds potentially exhibit fewer adverse effects than existing medications and may be useful as adjuvant or alternative to conventional antiplatelet medications. Capsaicin, the plant derived vanilloid, exerts its action through transient receptor potential vanilloid 1 (TRPV1) and TRPV1-independent mechanisms (Caterina and Julius, 2001). Along with other endogenous and exogenous vanilloid-like agents, capsaicin has been suggested to contain antiplatelet activity. However, the precise mechanism(s) of capsaicin action on platelets remain poorly understood. In this study the effects of plant-derived vanilloids; capsaicin and DHC, and endogenous vanilloids; OLDA and NADA on *in vitro* human platelet aggregation and their potential mechanism of action, have been investigated.

To the best of our knowledge, this is the first study to systematically investigate the effects of plant-derived (capsaicin and DHC) and endogenous vanilloids (OLDA and NADA) on platelet aggregation using an array of platelet agonists; ADP, collagen and AA. Furthermore, the potentially direct toxic effects of these compounds on platelets, and the impact on platelet function via alpha granule release, were investigated.

Capsaicin, OLDA and NADA significantly inhibited platelet aggregation induced by ADP in a concentration-dependent manner, while DHC had negligible effect on

ADP-induced aggregation. Interestingly, two previous studies have shown the same effect of capsaicin on *in vitro* human platelet aggregation induced by ADP (Adams et al., 2009, Raghavendra and Naidu, 2009). Although Adams et al. used the same *in vitro* aggregometry technique that has been used in the current study, they instead demonstrated that DHC enhanced the aggregation at low concentrations and inhibited at higher concentrations. The cause(s) for this is unknown but it might be due to different normal variation of the platelets donors.

Recently, it has reported that aggregation of canine platelets induced by collagen was completely inhibited by capsaicin (Mittelstadt et al., 2012). Furthermore, the same type of effect was demonstrated using human platelets (Raghavendra and Naidu, 2009). These finding are in contrast to those in the present study, where neither capsaicin nor DHC had any effect on the aggregation of human platelets induced by either 4 or 8µg/mL collagen. Moreover, the current study has demonstrated that the endovanilloids, OLDA and NADA, significantly inhibited platelet aggregation induced by 4µg/mL but not 8µg/mL collagen in a concentration-dependent manner. The reasons for the discrepant results across these studies are not known, however it may be due to the use of washed human platelets that were pre-incubated with capsaicin for five minutes prior to the addition of collagen (Raghavendra and Naidu, 2009) or species differences between the platelets from human compared to canine (Mittelstadt et al., 2012).

Capsaicin, DHC and NADA significantly inhibited the aggregation induced by 300 and 400 mg/mL AA and was concentration-dependent. The most potent vanilloid was capsaicin that inhibited the aggregation at 100µM by 90% followed by 50%

inhibition by 50 μ M. A similar effect of 60 μ M capsaicin has been reported on platelet aggregation induced by AA (Raghavendra and Naidu, 2009). As stated earlier it is not known why there are differences between the studies, but it might be due to the use of washed human platelets and/or pre-incubation with capsaicin for five minutes prior to the addition of AA. Nevertheless, capsaicin seems to interfere with the AA pathway.

The effects of capsaicin, DHC and other vanilloid-like agents on neuronal cells are mediated by both TRPV1-dependent and independent mechanisms (Caterina and Julius, 2001). To determine the contribution of TRPV1 activation toward the inhibitory effects of vanilloids on ADP-induced platelet aggregation, experiments were conducted in the presence of SB452533, a potent and selective TRPV1 antagonist. The inhibitory effects of capsaicin and OLDA on ADP-induced aggregation were not affected by SB452533, suggesting that the effects of these vanilloids were independent of TRPV1 activation. Although the present results are similar to those of a recent study that investigated the effect of TRPV1 antagonist (A-993610) on capsaicin's inhibition of collagen-induced aggregation of canine platelets (Mittelstadt et al., 2012), this does not exclude a role for CB receptors in the actions of either capsaicin or OLDA. The role of CB receptors in the inhibition of platelet aggregation remains to be investigated.

Vanilloids have been shown in many previous studies to be cytotoxic, particularly at higher concentrations, e.g., >25 μ M. In our laboratory for example, it has reported that 100 μ M NADA resulted in 100 percent cell death of human peripheral blood mononuclear cells (Saunders et al., 2009). Hence, to determine whether the

inhibition of platelet aggregation might be due to the direct platelet destruction by vanilloids, platelet LHD release following exposure to ADP, in the absence and presence of vanilloids, was measured. In the present study, four separate experiments were designed using three controls; background control (assay media), low control (cells only) and high control (100% cells lysis). The LDH level of vanilloids treated cells were the same as low control. None of the vanilloids tested (at up to 100 μ M) had any effect on LDH release. Hence, the inhibitory effects of vanilloids were not due to direct cytotoxicity.

Following confirmation that the vanilloid-like agents were not cytotoxic toward platelets, we investigated their effect on alpha granule release from platelets. Platelet alpha granules contain numerous haemostatic and non-haemostatic components (see chapter 1) that play a very important role in their function. PF4 and β -TG are synthesized only in megakaryocytes, and are secreted via exocytosis after platelet activation by strong and/or by weak agonists through TxA₂ generation. Protein kinase C, intracellular Ca⁺² and other factors regulate the fusion of alpha granule vesicles to the membrane (Reed, 2004, Reed et al., 2000). β -TG enhances neutrophil chemotaxis and adherence to endothelial cells (Brandt et al., 2000) and PF4 act as anticoagulant by binding to heparin with high affinity (Gupta and Singh, 1994). Accordingly, the effects of capsaicin, DHC, NADA and OLDA along with 10 μ M or 5 μ M ADP, on PF4 and β -TG release, were investigated. It was demonstrated that all vanilloids, with the exception of NADA, decreased the PF4 release and only capsaicin and OLDA decreased β -TG release.

It is important to note that the data presented has been generated by only two separate experiments, so this clearly needs to be further validated. However, it would appear that the preliminary data suggests that alpha granule release is affected by some vanilloids, especially OLDA and capsaicin. In comparison to the aggregation results, OLDA showed a strong effect on platelet aggregation induced by ADP and capsaicin too but to lesser extent comparing to OLDA. These effects might be through blocking of one and/or all ADP receptors directly, or by interfering with the intracellular signaling that leads to platelet secretion.

The precise mechanism/s of action of endogenous and plant-derived vanilloids on platelet aggregation remain to be elucidated, although numerous hypotheses have been proposed. A previous study showed that TRPV1 is present in platelets, and that capsaicin enhanced platelet aggregation in the absence of ADP or other platelet agonists, by increasing intracellular Ca^{+2} (Harper et al., 2009). Others have found that capsaicin rather inhibits cyclooxygenase1 (COX1), which may therefore result in a dampening of platelet aggregation (Raghavendra and Naidu, 2009). Furthermore, it has been suggested that capsaicin inhibits aggregation by inserting into platelet plasma membranes, changing its permeability and fluidity (Hogaboam and Wallace, 1991). A recent study has shown that capsaicin inhibited the aggregation of platelets and that it was not TRPV1 mediated (Mittelstadt et al., 2012). The results of the current study, using both endogenous and plant-derived vanilloids, strongly suggest that the inhibition of aggregation is not TRPV1 mediated, in agreement with these previous studies. The reason(s) for the contradictory results, i.e., activation vs inhibition, is unknown, however there are different possibilities. First, these studies used different techniques and platelets

preparation. Second, they used variant types of agonists and concentrations, also different concentrations of capsaicin and incubation times. Finally, it may be due to use of different platelet species and various volunteer criteria.

It was demonstrated that each vanilloid and endovanilloid/endocannabinoid produced different effects on platelet aggregation, depending on the agonist employed. These agonist-related differences may be due to different mechanisms of action and the different respective pathways of action. Collagen enhances cytosolic Ca^{2+} by binding directly to $\alpha_2\beta_1$ and GPVI, and indirectly through vWF $\alpha_{\text{Ib}}\beta_3$ and GPIb-IX-V, which activate granule release (ADP and serotonin) (Massberg et al., 2003, Kato et al., 2003). ADP instead binds to G-coupled protein receptors causing shape change, secretion, TxA_2 formation and aggregation, and also increases intracellular Ca^{2+} via the activation of PLC (Jin et al., 1998). AA is converted to TxA_2 via a series enzymatic reaction including COX1, increasing intracellular Ca^{2+} through activation of PLC (Nesbitt et al., 2003). Considering these pathways, it is interesting to speculate how the vanilloids investigated in this study interfere with platelets.

NADA was the only vanilloid to inhibit aggregation induced by all three agonists, and to the same degree. This effect might be through either direct blocking of CB1 and/or CB2 receptors, or non-specifically decreasing intracellular Ca^{2+} , or through unknown receptor/s. However, the effect of NADA as an endocannabinoid are opposite to those of Delta-9-tetrahydrocannabinol (THC), which activates human platelets by increasing P selectin and GPIIb/IIIa expression (Deusch et al., 2004). Furthermore, it increases 2-arachidonylglycerol (2-AG) levels, resulting in activation of platelets through the AA pathway (Nakahata, 2008). A further study has shown

that anandamide activates rabbit platelets through the same pathway (Braud et al., 2000). The previous studies showed that it is not CB1 or CB2 mediated. On the other hand, it has been reported that anandamide (arachidonylethanolamide, AnNE) activates human platelets through a mechanism independent of the AA pathway (Maccarrone et al., 1999). Later, the same group has shown that 2-AG mediated aggregation via an uncharacterized CB receptor (MacCarrone et al., 2001). The cause(s) for the conflicting results are uncertain, but it may be because each cannabinoid behaved differently. Moreover, these studies generated their results using different techniques, cannabinoids concentrations and incubation times.

The results of the current study suggest that OLDA may act through the blocking of the ADP receptors, P2Y1 and/or P2Y12, or by interfering/blocking of the intracellular ADP signaling pathway, as complete inhibition of aggregation induced by ADP was demonstrated. OLDA also damped aggregation mediated by collagen, with this effect completely inhibited by higher concentrations of collagen. This suggests that OLDA may be exerting its action through a non-receptor pathway, i.e., inserting directly into the phospholipid bilayer, or by partially antagonizing one or more collagen receptors. Although this inhibition was not through TRPV1, CB receptors and/or unknown receptors may be involved. Capsaicin and DHC completely inhibited aggregation induced by AA, which may be through the inhibition of COX1, in agreement with the previous study of (Raghavendra and Naidu, 2009). In addition, capsaicin also damped aggregation mediated by ADP, which may be due to partially antagonizing an ADP receptor or by impeding the intracellular signaling as capsaicin decreased alpha granule secretion. Moreover, the role of TRPV1 in capsaicin action has been excluded according to the results. The

nature of vanilloids and endovanilloids/endocannabinoids targets and actions on platelet remain to be elucidated.

Although this study has achieved most of its initial aims, there are number of limitations primarily due to time constraints. Firstly, the effects of vanilloids and endovanilloids/endocannabinoids on dense granule release, e.g., serotonin, were not completed. Secondly, the effects of TRPV1 antagonists with NADA, and the effects of CB1 and CB2 antagonists with vanilloids, on platelet aggregation, were also not investigated. Finally, alpha granule (PF4 and β -TG) data presented in this study is from only two experiments, and thus clearly needs to be confirmed.

Future directions include studies using whole blood aggregometry to determine the effects of endovanilloids/endocannabinoids on platelet aggregation, which more closely mimic *in vivo* conditions. Moreover, it would be useful to examine the effectiveness of endovanilloids/endocannabinoids in platelet hyperactivity disorders, such as the antiphospholipid syndrome, sticky platelet syndrome, myeloproliferative disorders, and others. Finally, the potential mechanism of action of endovanilloids/endocannabinoids, especially NADA, on platelet aggregation warrants further investigation. In this regard, Ca^{2+} flux studies, such as those performed by (Harper et al., 2009), would be useful.

In conclusion, the current study showed that exposure of platelets to capsaicin, DHC, NADA and OLDA inhibited platelet aggregation induced by ADP, collagen and AA albeit to different degrees. Additionally, the inhibitory effects of the vanilloids were not shown to be due to platelets destruction nor mediated by TRPV1 activation. In

addition, we found that vanilloids and endovanilloid might affect alpha granule secretion. The latter finding should be investigated further to find out at which cellular level this effect is occurring. Vanilloids and endovanilloids/endocannabinoids have potent antiplatelet activity and should be further investigated as potential antiplatelet and/or adjuvant with antiplatelet medications.

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